

# Analytical Profiles of Drug Substances

Volume 14

*Edited by*

Klaus Florey

**Analytical Profiles  
of  
Drug Substances**

Volume 14

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**Klaus Florey**

The Squibb Institute for Medical Research  
New Brunswick, New Jersey

Contributing Editors

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*Compiled under the auspices of the  
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APhA Academy of Pharmaceutical Sciences*



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# CONTENTS

<i>Affiliations of Editors, Contributors, and Reviewers</i>	vii
<i>Preface</i>	ix
 Chlorthalidone	 1
<i>Jeff M. Singer, Michael J. O'Hare, Carl R. Rehm, and     John E. Zarembo</i>	
 Imipramine Hydrochloride	 37
<i>Donald N. Kender and Richard E. Schiesswohl</i>	
 Cisplatin	 77
<i>Christopher M. Riley and Larry A. Sternson</i>	
 Tripelethamine Hydrochloride	 107
<i>Hazel G. Piskorik</i>	
 Xylometazoline Hydrochloride	 135
<i>Yechiel Golander and Wayne J. DeWitte</i>	
 Mefloquine Hydrochloride	 157
<i>Peter Lim</i>	
 Iopanoic Acid	 181
<i>Davide Pitre</i>	
 Lidocaine Base and Hydrochloride	 207
<i>K. Gröningsson, J.-E. Lindgren, E. Lundberg,     R. Sandberg, and A. Wahlén</i>	

Benperidol	245
<i>Pamela G. Takla, Kenneth C. James, and Ahmed Eliass H. Gassim</i>	
Terpin Hydrate	273
<i>Mahmoud M.A. Hassan, Jaber S. Mossa, and Abdul Hameed U. Kader Taragan</i>	
Atropine	325
<i>Abdullah A. Al-Badr and Farid J. Muhtadi</i>	
Isoproterenol	391
<i>Mohammad Tariq and Abdullah A. Al-Badr</i>	
Warfarin	423
<i>Salim A. Babhair, Mohammad Tariq, and Abdullah A. Al-Badr</i>	
Naloxone Hydrochloride	453
<i>Mahmoud M.A. Hassan, Mohamed E. Mohamed, and Mohammad Saleem Mian</i>	
Diffunisal	491
<i>Meredith Cotton and Robert A. Hux</i>	
Baclofen	527
<i>Satinder Ahuja</i>	
PROFILE SUPPLEMENTS	
Acetaminophen	551
<i>Humeida A. El-Obeid and Abdullah A. Al-Badr</i>	
Halothane	597
<i>Chester E. Orzech and Frederick Q. Gemmill, Jr.</i>	
Cumulative Index	619

## AFFILIATIONS OF EDITORS, CONTRIBUTORS, AND REVIEWERS

- S. Ahuja*, CIBA-GEIGY Corporation, Suffern, New York  
*S. A. Babhair*, King Saud University, Riyadh, Saudi Arabia  
*A. A. Al-Badr*, King Saud University, Riyadh, Saudi Arabia  
*S. A. Benezra*, Wellcome Research Laboratories, Research Triangle Park,  
North Carolina  
*R. Bishara*, Lilly Research Laboratories, Indianapolis, Indiana  
*G. S. Brenner*, Merck Sharp & Dohme Research Laboratories, West Point,  
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*M. Cotton*, Merck Frosst Canada Inc., Pointe Claire-Dorval, Quebec,  
Canada  
*N. DeAngelis*, Wyeth Laboratories, Philadelphia, Pennsylvania  
*W. J. DeWitte*, CIBA-GEIGY Corporation, Suffern, New York  
*H. A. El-Obeid*, King Saud University, Riyadh, Saudi Arabia  
*J. E. Fairbrother*, Stiefel Laboratories Ltd., Sligo, Ireland  
*K. Florey*, The Squibb Institute for Medical Research, New Brunswick,  
New Jersey  
*A. E. H. Gassim*, The Welsh School of Pharmacy, Cardiff, Wales  
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*K. Gröningsson*, Astra Läkemedel AB, Södertälje, Sweden  
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*E. Lundberg*, Astra Läkemedel AB, Södertälje, Sweden

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*H. G. Piskorik*, CIBA-GEIGY Corporation, Suffern, New York  
*D. Pitre*, Bracco Industria Chimica s.p.a., Milan, Italy  
*C. R. Rehm*, Revlon Health Care Group, Tuckahoe, New York  
*C. M. Riley*, University of Florida, Gainesville, Florida  
*R. Sandberg*, Astra Läkemedel AB, Södertälje, Sweden  
*R. E. Schiesswohl*, CIBA-GEIGY Corporation, Suffern, New York  
*J. M. Singer*, Revlon Health Care Group, Tuckahoe, New York  
*L. A. Sternson*<sup>1</sup>, University of Kansas, Lawrence, Kansas  
*P. G. Takla*, The Welsh School of Pharmacy, Cardiff, Wales  
*A. H. U. K. Taragan*, King Saud University, Riyadh, Saudi Arabia  
*M. Tariq*, King Saud University, Riyadh, Saudi Arabia  
*A. Wahlén*, Astra Läkemedel AB, Södertälje, Sweden  
*J. E. Zarembo*, Revlon Health Care Group, Tuckahoe, New York

<sup>1</sup>Present address: Smith Kline & French Laboratories, Philadelphia, Pennsylvania.

## PREFACE

The compilation of *Analytical Profiles of Drug Substances* to supplement the information contained in the official compendia is now a well-established activity.

That we are able to publish one volume per year is a tribute to the diligence of the editors to solicit articles and even more so to the enthusiastic response of our authors, an international group associated with pharmaceutical firms, academic institutions, and compendial authorities. I would like to express my sincere gratitude to them for making this venture possible.

Over the years, we have had queries concerning our publication policy. Our goal is to cover all drug substances of medical value, and therefore, we have welcomed any papers of interest to an individual contributor. We also have endeavored to solicit profiles of the most useful and used medicines, but many in this category still need to be profiled.

In the preface to the eleventh volume, I announced that we would try to supplement previously published profiles with new data. Unfortunately, most of the original contributors are no longer available to undertake this task, and it has proven difficult to find other volunteers. We shall continue to pursue the updating program, but it will not be as comprehensive as originally envisioned.

Again, I would like to request those who have found these profiles useful to contribute papers of their own. We, the editors, stand ready to receive such contributions.

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# CHLORTHALIDONE

Jeff M. Singer, Michael J. O'Hare,  
Carl R. Rehm, and John E. Zarembo

*Revlon Health Care Group  
Tuckahoe, New York*

Introduction	2
1. Description	2
1.1 Name, Formula, Molecular Weight, Chemical Names	2
1.2 Trade Names	3
1.3 Appearance, Color	3
2. Physical Properties	3
2.1 Spectra	3
2.2 Physical Properties of the Solid	14
2.3 Solubility	17
2.4 Ionization Constant (pKa)	21
3. Synthesis	21
4. Stability	21
5. Chemical Properties	22
5.1 Identity Tests	22
5.2 Methods of Analysis	22
5.3 Chromatographic Analysis	25
6. Determination in Biological Fluids	30
7. Bioavailability and Pharmacokinetics	32
References	34



## INTRODUCTION

Chlorthalidone is an antihypertensive diuretic used in the treatment of edema associated with congestive heart failure. It is the active component in Hygroton<sup>®</sup>, Regroton<sup>®</sup> and Demi-Regroton. The drug shows long lasting diuretic actions similar to those of other thiazide diuretics such as chlorothiazide. It is absorbed slowly from the gastrointestinal tract and is excreted largely as unchanged drug. The overall duration of effect is 48 to 72 hours.

## ANALYTICAL METHODS FOR ANALYSIS OF CHLORTHALIDONE

### 1. DESCRIPTION

#### 1.1 Name, Formula, Molecular Weight, Chemical Names

Chlorthalidone is designated by the following names:

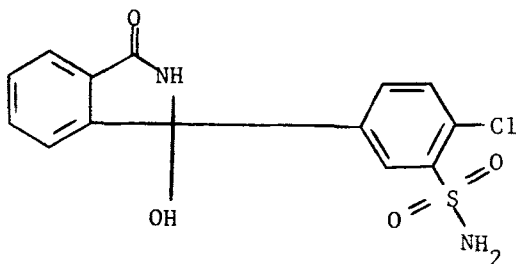
Benzenesulfonamide, 2-Chloro-5-(2,3 dihydro-1-hydroxy-3-oxo-1H-isoindol-1-yl)

2-Chloro-5'-(1-hydroxy-3-oxo-1-isoindolinyl)  
benzene sulfonamide

and is also known as:

3-Hydroxy-3-(4-chloro-3-sulfamylphenyl)  
phthalimidine.

The empirical formula is  $C_{14}H_{11}ClN_2O_4S$  with a molecular weight of 338.76.



## 1.2 Trade Names

Hygroton<sup>®</sup>  
Regroton<sup>®</sup>  
Hydro-Long  
Hydroton  
Igroton

Chlorthalidone is also a component of Regroton<sup>®</sup> and Demi-Regroton combination products containing Chlorthalidone in combination with reserpine.

## 1.3 Appearance, Color

Chlorthalidone is a white to yellowish-white crystalline powder.

## 2. PHYSICAL PROPERTIES

### 2.2 Spectra

#### 2.1.1 Infrared Spectrum

The infrared absorption spectrum of Chlorthalidone obtained from a potassium bromide dispersion is shown in Figure 1. The spectrum was recorded on a Perkin-Elmer 621 Grating Infrared Spectrophotometer. Table 1 contains the assignments of several of the characteristic absorption bands.

#### 2.1.2 Mass Spectrum

The mass spectrum of Chlorthalidone is presented in Figure 2. A Varian MAT-112 mass spectrometer, operating in the electron impact ionization mode at 70 eV and using a source and probe temperature of 250°C, was used for the analysis. The peak assignments are listed in Table 2. The base peak is at m/z 148. These results are in agreement with the previously published work of Frigerio and Pantarotto (1).

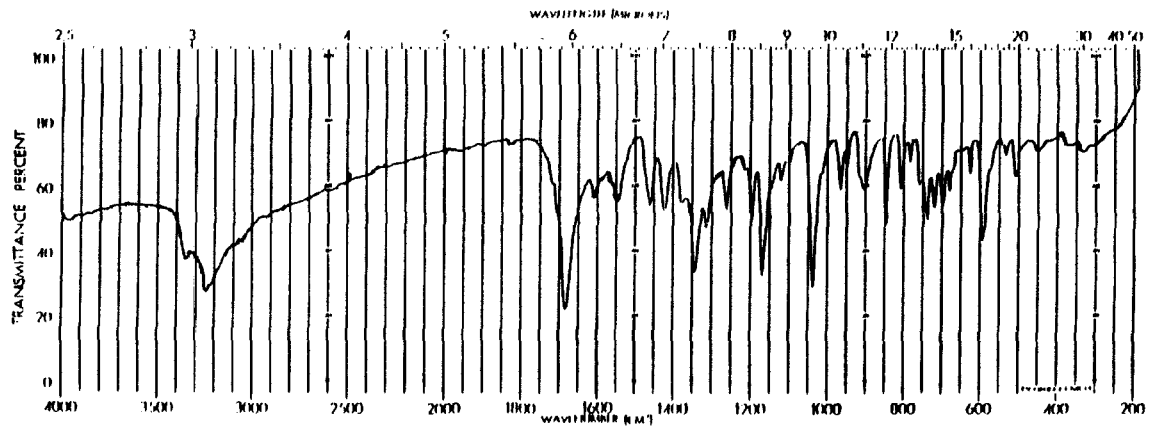


FIGURE 1

INFRARED SPECTRUM OF CHLORTHALIDONE: KBr DISPERSION

INSTRUMENT: PERKIN-ELMER 621

TABLE 1

INFRARED BAND ASSIGNMENTS FOR CHLORTHALIDONE

<u>BAND (cm<sup>-1</sup>)</u>	<u>INTENSITY*</u>	<u>ASSIGNMENT</u>
3350	M	O-H Stretch
3240	M	N-H Stretch
1685	S	$\begin{array}{c} \text{O} \\    \\ \text{-C-NH Amide} \end{array}$
1345	S	Sulfonamide
1170	S	Sulfonamide
1038	S	O-H bending
595	S	C-Cl Stretch

\* M = Medium

S = Strong

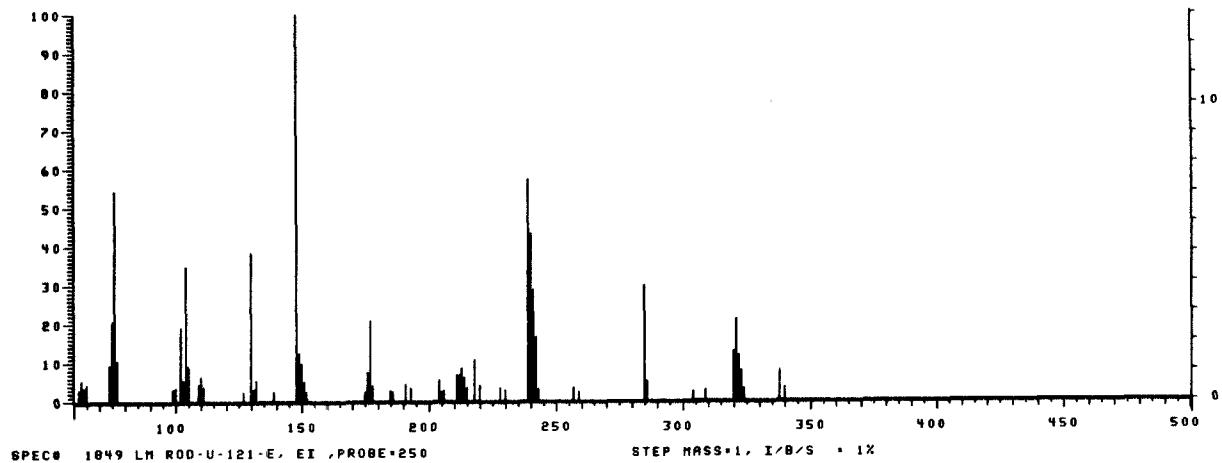
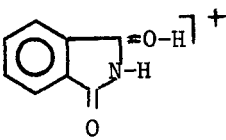
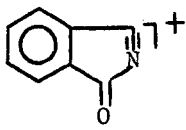
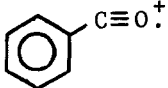
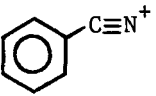


FIGURE 2

MASS SPECTRUM OF CHLORTHALIDONE, ELECTRON IMPACT IONIZATION  
INSTRUMENT: VARIAN MAT-112

TABLE 2

MASS SPECTRUM OF CHLORTHALIDONE

<u>PEAK #</u>	<u>MASS #</u>	<u>REL. INT.</u>	<u>ASSIGNMENT</u>
1	340	2.4	$M^+ + 2$ =(indicates 1 chlorine)
2	338	6.5	$M^+$ (Molecular ion)
3	321	14.6	$M^+ - OH\cdot$
4	285	27.4	$M^+ - H_2O - Cl$
5	239	67.9	$M^+ - H_2O - SO_2NH_2$
6	177	19.0	Not Assigned
7	148	100.0	
8	130	36.4	
9	104	43.7	
10	102	18.0	

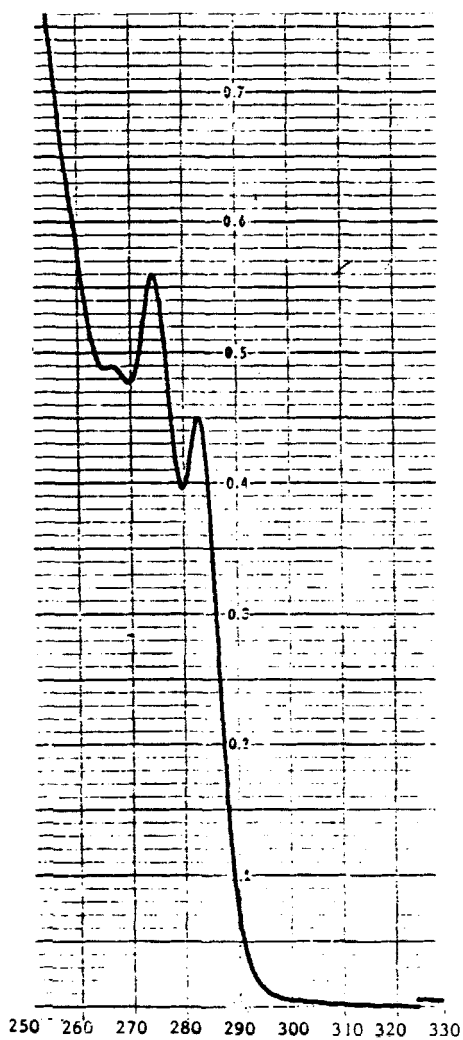


FIGURE 3      ULTRAVIOLET SPECTRUM OF CHLORTHALIDONE  
INSTRUMENT: CARY 219

### 2.1.3 Ultraviolet Absorption Spectrum

A Cary 219 Spectrometer was used to record the ultraviolet spectrum of Chlorthalidone in dilute HCl/methanol, 1:50 (v:v). The spectrum in Figure 3 shows an aromatic multiplet with maxima at 266 nm ( $\epsilon=4.73$ ), 275 nm ( $\epsilon=5.40$ ) and 283 nm ( $\epsilon=4.33$ ).

### 2.1.4 Nuclear Magnetic Resonance Spectrum

The proton spectrum shown in Figure 4 was obtained on a JEOL FX 90Q NMR Spectrometer using DMSO- $d_6$ . The band assignments are referenced to a TMS internal standard and are listed in

Table 3. Singlets occur at 9.38 ppm and 7.20 ppm due to protons "a" and "b" respectively. Protons "a", "b" and "e" are exchangeable with the addition of D<sub>2</sub>O. A doublet due to the proton assigned as "d" occurs at 8.11 ppm.

Multiplets at 7.63-7.20 ppm and 7.63-7.48 ppm are due to the protons assigned as "c" and "e". The additional peaks in the spectrum are due to solvents.

The completely proton decoupled 22.5 MHz <sup>13</sup>C spectrum in DMSO- $d_6$  has also been recorded and is shown in Figure 5; DMSO- $d_6$  was used as a reference. Chemical shifts and multiplicities are compiled in Table 4. The signal at 168.4 ppm has been assigned to the amide carbon, C-7; the signal at 86.8 ppm has been assigned to the hydroxylated carbon, C-8. The remaining peaks, occurring within the range 149.8 - 122.7 ppm are due to the aromatic carbons.



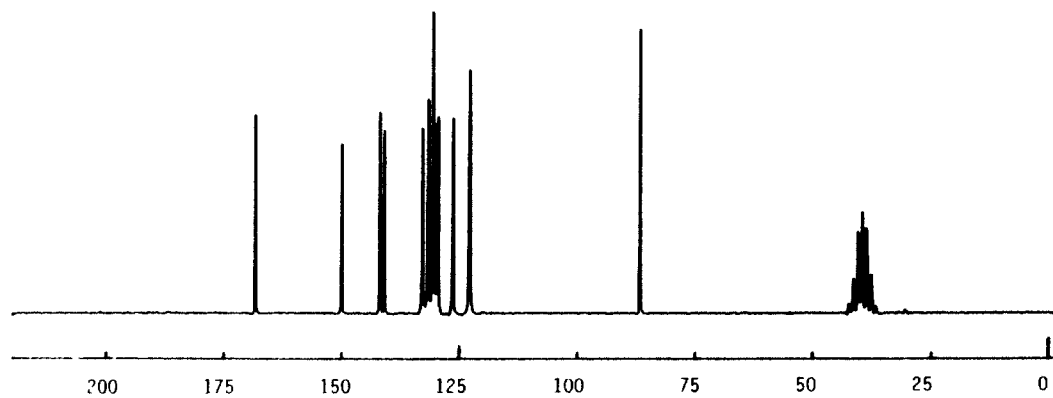
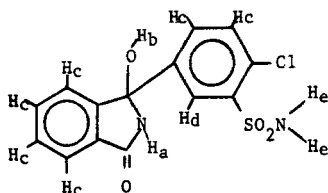


FIGURE 5: THE  $^{13}\text{C}$  NMR SPECTRUM OF CHLORTHALIDONE IN  $\text{DMSO-d}_6$  WITH DMSO AS INTERNAL STANDARD  
INSTRUMENT: JEOL FX 90Q

TABLE 3

90 MHz <sup>1</sup>H NMR ANALYSIS OF CHLORTHALIDONE

<u>PROTON</u>	<u># OF H's</u>	<u>CHEMICAL SHIFT (ppm)</u>	<u>MULTIPLICITY</u>	<u>J(Hz)</u>	<u>D<sub>2</sub>O EXCHANGEABILITY</u>
a	1	9.38	Singlet		Y
b	1	7.20	Singlet		Y
c	6	7.63 - 7.20	Multiplet		N
d	1	8.11	Doublet	0,5	N
e	2	7.63 - 7.48	Multiplet		Y

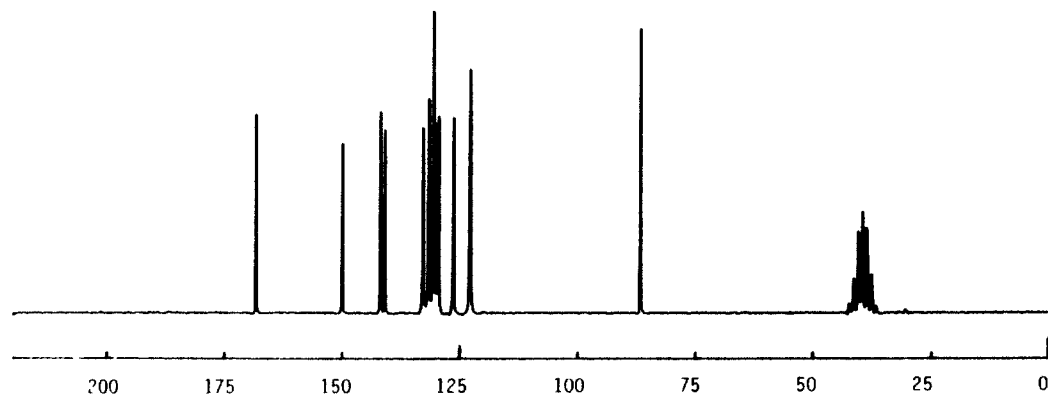
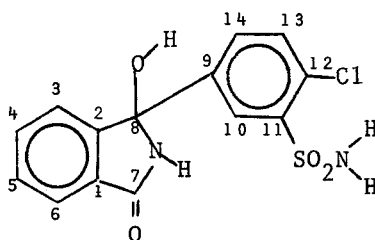


FIGURE 5: THE  $^{13}\text{C}$  NMR SPECTRUM OF CHLORTHALIDONE IN  $\text{DMSO-d}_6$  WITH DMSO AS INTERNAL STANDARD  
INSTRUMENT: JEOL FX 90Q

TABLE 4

22.5 MHz  $^{13}\text{C}$  NMR ANALYSIS OF CHLORTHALIDONECHEMICAL SHIFTMULTIPLICITY\*

168.4	S
149.9	S
141.9	S
140.9	S
132.8	D
131.6	D
130.6	-
130.0	-
129.4	D
126.3	D
122.9	D
122.7	D
86.8	S

\* Off-Resonance

S = Singlet

D = Doublet

## 2.2 Physical Properties of the Solid:

### 2.2.1 Melting Range (MR)

Laboratory investigations have shown that a sample of Chlorthalidone melts in the range of 215°-222°C, followed by decomposition above this region. This contrasts with the range of 224-226°C (with decomposition) cited in the Merck Index (2). It is also stated that the melting range may be extended from 218-264°C upon slow heating. These melting ranges have been confirmed using a Leitz HM-POL Microscope equipped with a Mettler SP52 Hot Stage and Mettler FP5 Controller. A heating rate of 10°C/min was shown to produce the narrower range while a heating rate of 2°C/min agreed with the extended range.

### 2.2.2 Thermogravimetric Analysis (TGA)

Thermogravimetric analysis of Chlorthalidone was performed on a Perkin-Elmer TGS-2 Thermogravimetric Analyzer. The TGA curve is shown in Figure 6. The compound was heated from 40°C to 600°C at a rate of 20°C/min.

A gradual weight loss begins at about 200°C and results in an indistinct plateau from about 255°C to 290°C, with a weight loss of approximately 5%, corresponding to a loss of 1 mole of water. Above this temperature the weight loss is gradual without any plateaus.

### 2.2.3 Differential Scanning Colorimetry (DSC)

A DSC thermogram of Chlorthalidone is shown in Figure 7. The endotherm was obtained on a Perkin-Elmer DSC-2C

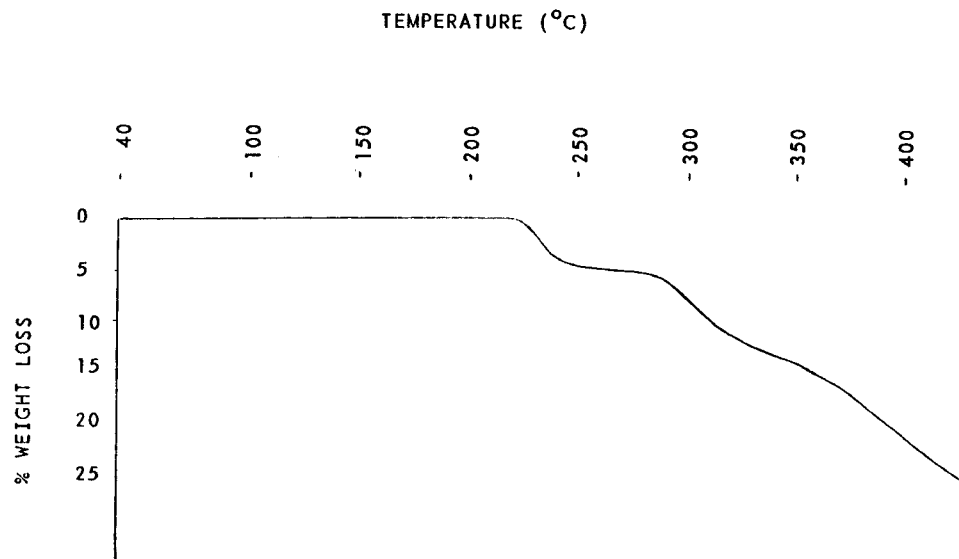


FIGURE 6: THE TGA THERMOGRAM OF CHLORTHALIDONE  
INSTRUMENT: PERKIN-ELMER TGS-2

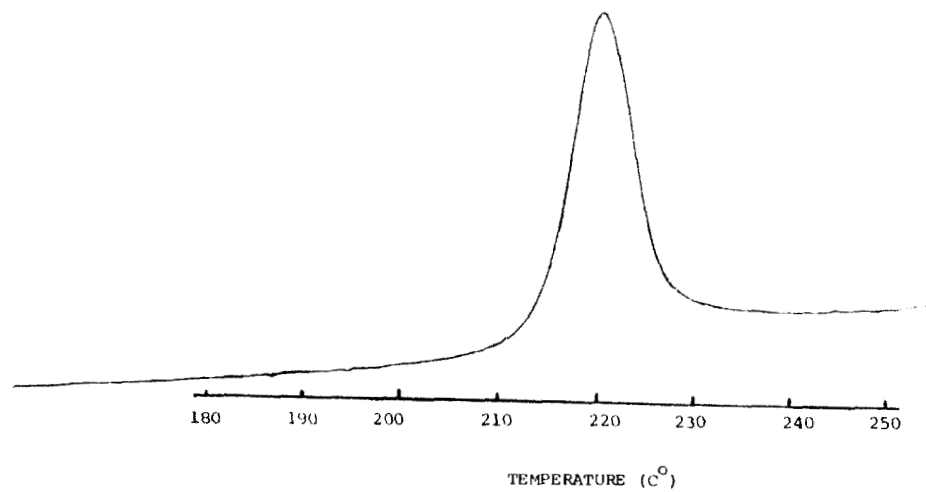


FIGURE 7: THE DSC THERMOGRAM OF CHLORTHALIDONE  
INSTRUMENT: PERKIN ELMER DSC-2C

Differential Scanning Calorimeter at a heating rate of 10°C/min. over the range of 50°C to 230°C. From the endothermic peak a "melting point" of 214°C was calculated. Upon cooling the sample down to 170°C and reheating up to 230°C, no melting endotherm was observed. This is due to the formation of an amorphous glass.

#### 2.2.4 Polymorphism

Chlorthalidone was studied in these laboratories by infrared, x-ray powder diffraction, differential scanning calorimetry and optical hot stage polarized light microscopy to determine if any polymorphic modifications were produced when Chlorthalidone was recrystallized from DMF/H<sub>2</sub>O, acetic acid, acetone and other polar and non-polar solvents. Only one single crystalline form was produced.

### 2.3 Solubility

#### 2.3.1 Solubility in Water

The solubility of Chlorthalidone in water has been measured spectrophotometrically as a function of pH. The pH solubility profile of Chlorthalidone, determined at room temperature, increased steadily from 16.7 mg/100 mL at pH 4.90 to 59.7 mg/100 mL at pH 9.60. Above pH 10.0 the solubility dramatically increases to a value of 991.1 mg/100 mL at pH 10.9 (see Table 5) due to salt formation.



TABLE 5

SOLUBILITY OF CHLORTHALIDONE AS A FUNCTION OF pH  
(ROOM TEMPERATURE)

<u>pH</u>	<u>SOLUBILITY OF CHLORTHALIDONE</u> <u>mg/mL SATURATED SOLUTION</u>
4.90	0.167
7.00	0.180
7.70	0.183
8.40	0.210
8.65	0.230
8.95	0.300
9.40	0.390
9.60	0.597
10.00	1.201
10.10	2.958
10.30	4.698
10.50	5.534
10.90	9.911

<u>Temperature</u> (°C)	<u>Solubility in</u> <u>Water(mg/100 mL)</u>	<u>Solubility in</u> <u>0.1N Na<sub>2</sub>CO<sub>3</sub></u> <u>(mg/100 mL)</u>
20°	12	577
37°	27	990

### 2.3.2 Solubility in Water-Miscible Solvents

The solubility of Chlorthalidone in a variety of solvents has been determined. Chlorthalidone has been found to be freely soluble in dimethylacetamide (DMA), dimethylformamide (DMF), dimethylsulfoxide (DMSO) and methanol. The solubility reported for Chlorthalidone in ethanol varies from slightly soluble at room temperature to soluble in warm ethanol.

### 2.3.3 Solubility in Water-Immiscible Solvents

Chlorthalidone has been found to be insoluble in chloroform and diethyl ether at room temperature.

### 2.3.4 Solubility in Oils of Pharmaceutical Interest

Solubilization of Chlorthalidone in polyethylene glycol (PEG 200 and PEG 400) occurs slowly.

Spectrophotometric determination of the solubility of Chlorthalidone in varying ratios of PEG 400 and water (0%, 20%, 40%, 60%, 80%, 100% (v/v) showed that there is a 1000-fold increase in the solubility of chlorthalidone in PEG 400 as compared to water (see Table 6).

TABLE 6

SOLUBILITY OF CHLORTHALIDONE AS A FUNCTION OF  
VOLUME % PEG 400

<u>VOLUME % PEG 400</u>	<u>SOLUBILITY OF CHLORTHALIDONE</u> <u>mg/mL SATURATED SOLUTION*</u>
0	0.13
20	0.98
40	4.22
60	18.6
80	67.8
100	141.5

\*After 65 hour shaking at room temperature.

#### 2.4. Ionization Constant (pKa)

The sulfonamide function present in Chlorthalidone, is considered to be responsible for the acid dissociation (3). The ionization constant of Chlorthalidone was determined based on spectrophotometric measurements (at 275 nm) of the concentration at various pH values. A single pKa value of 9.36 in water (22°+ 1°C) was obtained, which indicates that Chlorthalidone is a weakly mono-acidic compound. This value compares favorably with benzenesulfonamide, pKa = 10.0 (4). Potentiometric difference titrations produced a value of  $9.24 \pm 0.02$  in 0.1M aqueous KCl which, when corrected for ionic strength, yields a thermodynamic dissociation constant of 9.35 (25°C) (3), which is in excellent agreement with the spectrophotometric determination.

#### 3. SYNTHESIS AND PURIFICATION

3-Amino-4-chlorobenzophenone-2-carboxylic acid is diazotized and the resulting diazonium chloride is reacted in the cold with sulfur dioxide in glacial acetic acid in the presence of cupric chloride to form 4-chloro-2'-carboxy-benzophenone-3-sulfonyl chloride (I). Heating I with thionyl chloride yields 3-chloro-3-(3'-chloro-sulfonyl-4'-chloro-phenyl) phthalide which is isolated, dissolved in chloroform, and reacted with ammonia in the cold in the presence of ethanol. Removal of the solvent and treatment of the residue with HCl yields crude Chlorthalidone which is recrystallized from aqueous ethanol. US Pat 3,055,904.

#### 4. STABILITY

Chlorthalidone is stable as a dry powder for a minimum of 3 years. No degradation of the active ingredient, after storage at room temperature, was detected.

## 5. CHEMICAL PROPERTIES

### 5.1 Identity Tests

Chlorthalidone may be identified by reaction with concentrated sulfuric acid to yield an intense yellow color.

Identification may also be made on the basis of measurement of the infrared and ultraviolet absorption spectra and comparison with USP reference spectra. HPLC retention time and TLC, Rf values, may also be used for identification purposes (*vide infra*).

### 5.2 Methods of Analysis

#### 5.2.1 Elemental Analysis: C,H,N

The results of CHN analysis of Chlorthalidone USP, Reference Standard performed on a Perkin-Elmer 240B Elemental Analyzer are shown below:

<u>ELEMENT</u>	<u>% THEORY</u>	<u>FOUND</u>
Carbon	49.64	49.86
Hydrogen	3.27	3.56
Nitrogen	8.27	8.12

#### 5.2.2 Acidity

The acidity of Chlorthalidone may be measured potentiometrically, in accordance with USP procedure (5). This measures residual acetic acid and/or Chlorthalidone carboxylic acid and which could be present in trace amounts. An accurately weighed 1.0 gm sample of

Chlorthalidone is dissolved in 25 mL of heated p-dioxane. After cooling, the sample is diluted to 50 mL with distilled water, and titrated potentiometrically, under nitrogen, with 0.1N NaOH using a glass/ calomel electrode in combination with a Metrohm E636 Microprocessor unit. A blank is determined similarly. Not more than 1.2 mLs of 0.1N NaOH is required to neutralize a 1.0 gm sample of Chlorthalidone.

### 5.2.3 Non-Aqueous Titrimetric Analysis with TBAH

The non-aqueous titration of Chlorthalidone is the preferred procedure due to the weak acidity of the compound. This is been performed potentiometrically with 0.1N tetrabutyl-ammonium hydroxide VS in anhydrous methanol\* (standardized against benzoic acid) using a glass/calomel electrode (with a salt bridge of 0.01N tetrabutylammonium bromide in methanol). The analysis is performed under a nitrogen atmosphere and the buret delivering the titrant is equipped with a carbon dioxide absorption trap. An accurately weighed 200 mg sample is dissolved in 50 mLs of acetone and titrated potentiometrically to the end point with a Metrohm E636 Microprocessor unit; a blank of 50 mLs of acetone is also determined.

$$\% \text{ Chlorthalidone} = \left[ \frac{V_1 N_1}{S_1} - \frac{V_2 N_2}{S_2} \right] \times 33.88$$

Where:  $V_1$  = volume, in mLs  
(corrected for blank), of TBAH  
required for the Titrimetric  
Assay

$N_1$  = normality of TBAH used  
in the Titrimetric Assay

$S_1$  = sample wt, in gms, used  
in Titrimetric Assay (dried  
basis)

$V_2$  = volume, in mLs  
(corrected for blank), of NaOH  
required for the Acidity  
Determination

$N_2$  = normality of NaOH used  
in the Acidity Determination

$S_2$  = sample wt, in gms, used  
in the Acidity Determination  
(dried basis)

\* USP XX also indicates TBAH may be made up in toluene:  
methanol, 9:1.

#### 5.2.4 Ultraviolet Spectrophotometric Analysis

The ultraviolet absorption of a solution of Chlorthalidone in 2N hydrochloric acid in methanol at 275 nm has been used for the quantitative analysis of the drug in dosage forms (5). A sample, equivalent to 400 mg of Chlorthalidone, is extracted into acetone, digested, filtered, evaporated to dryness, and diluted with an appropriate volume of methanol for comparison to a standard solution. The method is sensitive to a concentration of 100 ug/mL.

### 5.3 Chromatographic Analysis

#### 5.3.1 High Performance Liquid Chromatography (HPLC)

A liquid chromatographic method has been developed to allow the quantitative determination of Chlorthalidone in pharmaceutical dosage forms and active drug substance (6). The procedure employs a solvent extraction with acetonitrile-water (9:1 v/v) to liberate the active ingredient from the tablet material. Analysis was performed on a DuPont Model 830 HPLC equipped with a 254 nm UV absorption detector. A 1 m x 2 mm i.d. stainless steel column packed with a polyamide-coated stationary phase (Pellamidon, Whatman) was used for the analysis. The mobile phase of 2-propanol:acetic acid:water: n-hexane (30:1.5:0.5:68 v/v) at a flow rate of 2 mL/min is used. Quantitation was accomplished by comparison with an external standard solution prepared with the reference standard. A typical HPLC chromatogram is displayed in Figure 8, showing the resolution of Chlorthalidone from the expected hydrolysis product, chlorthalidone carboxylic acid (CCA). An alternate HPLC method employing different operating parameters has also been used successfully for the assay of Chlorthalidone in tablets (7). The active ingredient is extracted into methanol, mixed with an internal standard (2,7-dihydroxynaphthalene) and diluted to an appropriate volume with water. Analysis is performed on a 4.6 mm x 25 cm column packed with either Zorbax Cg (DuPont) or Partisil PxSCg (Whatman)



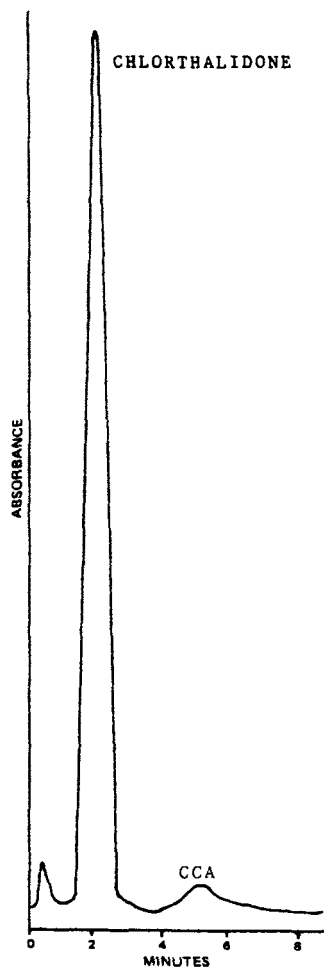


FIGURE 8

REPRESENTATIVE HIGH PRESSURE LIQUID CHROMATOGRAM  
INSTRUMENT: DUPONT 830

using a mobile phase consisting of 0.01 M dibasic ammonium phosphate and methanol (3:2 v/v), adjusted to pH 5.5 with phosphoric acid; the flow rate is maintained at 1 ml/min. Detection is accomplished with a 254 nm UV absorption detector. The method is also capable of resolving Chlorthalidone from the hydrolysis product, CCA. This method has supplanted the titrimetric assay (Sect. 5.2.3) as the compendial method (8). Other investigators have utilized various C<sub>18</sub> columns for the analysis of Chlorthalidone, either alone (9) or in combination with other drugs (10).

### 5.3.2 Gas Liquid Chromatography

Direct analysis by gas chromatography of underivatized Chlorthalidone is not possible, due to the three polar active groups contained within the molecule. However, conversion of Chlorthalidone to its tetramethyl derivative by extractive alkylation with tetrahexyl-ammonium hydrogen sulfate produces a molecule with suitable chromatographic properties (11). This procedure allows chromatographic analysis on a 1.7 m x 2 mm i.d. glass column packed with 3% JXR on 100/120 mesh Gas Chrom Q. Detection was accomplished with <sup>63</sup>Ni electron capture detector. The procedure proved suitable for the determination of nanogram quantities of the drug in plasma. Other investigators have employed other silicone liquid phases to effect separation: 3% OV-1 on 80/100 mesh Supelcoport<sup>(12)</sup>, 3% QF-1 on 100/120 mesh Gas Chrom Q<sup>(13)</sup>, and 1% SE-30 on 80/100 Gas-Chrom Q<sup>(13)</sup>. This method

was later modified by Fleuren and van Rossum (14), with greatly improved sensitivity, utilizing a nitrogen-specific detector.

Subsequent commercial unavailability of the tetrahexylammonium hydrogen sulfate prompted the development an on-column methylation procedure (15). This method employs a 0.2 M solution of trimethylanilinium hydroxide in methanol as the derivatizing agent, after Brochmann-Hanssen and Oke (16). In addition, the Chlorthalidone analog, 3-isobutyloxy-3-(3'-Sulfonamido-4'-chlorophenyl) phthalimidine, demonstrated suitability for use as an internal standard (see Figure 9 for a representative chromatogram). The analysis was performed on a Perkin-Elmer 900 gas chromatograph equipped with a flame ionization detector, using the following instrument parameters:

Column: 1.8 m x 2 mm i.d. glass,  
packed with 3% JXR on 100/120  
mesh Gas-Chrom Q.

Column Temperature: 190°C, temperature  
programmed at  
4°C/min. to 260°C

Injector Temperature: 300°C

Detector Temperature: 300°C

Carrier/Flow Rate: Helium at 40 cc/min.

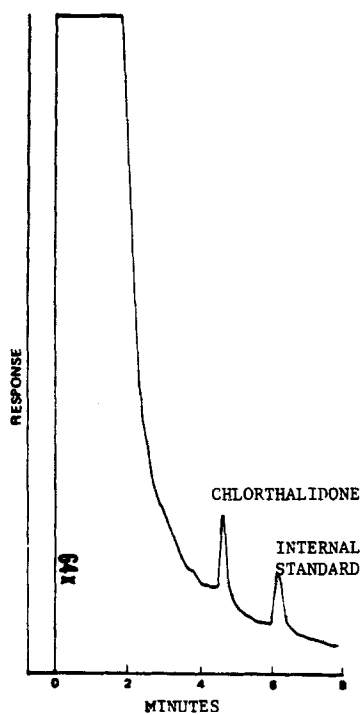


FIGURE 9 GAS CHROMATOGRAM OF A SPIKED CONTROL HUMAN URINE SAMPLE  
AFTER ON-COLUMN METHYLATION  
INSTRUMENT: PERKIN-ELMER 900

### 5.3.3 Thin Layer Chromatography

The analysis of pharmaceutical dosage forms for Chlorthalidone by thin layer chromatography is accomplished by the separation of possible impurities, such as chlorthalidone carboxylic acid (CCA). A 5% solution of the sample was prepared in acetone/water (9:1 v/v) and a 500 ug aliquot was applied to a Silica Gel GF plate (Merck, EM 5765, SG60, F254, 250 u, 20 x 20 cm). The developing solvent consisted of ethyl acetate: absolute ethanol: concentrated ammonium hydroxide (50:20:30 v/v). A chromatography tank lined with U-shaped Whatman #1 paper was equilibrated for 3 hours prior to use. The plate was developed to a height of 15 cm. After air-drying, the plate was observed under shortwave ultraviolet light to determine the Rf values. Chlorthalidone chromatographs to an Rf of 0.60; CCA has an Rf value of 0.46.

## 6. DETERMINATION IN BIOLOGICAL FLUIDS

The distribution of  $^{14}\text{C}$ -labelled Chlorthalidone was monitored via liquid scintillation counting of tissues, blood, bile and excretion following intravenous or oral administration (17). The method, however, lacked specificity since the total radioactivity of the metabolites and unmetabolized Chlorthalidone was measured. Solvent extraction, followed by deamination of Chlorthalidone in alkali was used by Pulver et. al. (18) as the basis for the spectrophotometric assay at 263 nm of the drug in blood, urine and animal tissues. The method allowed the determination of quantities greater than, approximately, 10 mg/L. Tweeddale and Ogilvie (19) improved the technique by varying the initial extraction conditions and amount of alkali used to allow a detection limit of 1 mg/L at 262 nm.

Fleuren and van Rossum (14) have described a sensitive and selective gas chromatographic method for determination of the drug in plasma, urine and red blood cells, utilizing derivatization via extractive alkylation. The on-column methylation procedure of Li et. al. (15), after Brochmann-Hanssen and Oke (16), is sensitive for the determination of urinary Chlorthalidone levels of 100 ng/mL.

The ability of Chlorthalidone to inhibit the enzymatic hydrolysis rate of p-nitrophenyl acetate by bovine erythrocyte carbonic anhydrase is the basis for the spectrophotometric assay of urinary samples (20). Measurement of the absorbance at 400 nm monitors the formation of the p-nitrophenol which is proportional to the drug concentration. The method was subsequently automated (21), utilizing continuous flow equipment, to extract the urinary samples and perform the enzymatic reaction, with a sensitivity of 0.5 ug/mL. The analysis of plasma concentrations, via this method necessitated manual extraction prior to automated analysis. The optimum sensitivity allowed the detection of 25 ng/mL.

Guelen et. al. (22) have developed an HPLC method for the analysis of Chlorthalidone in human blood, plasma and urine. The authors employed an octadecylsilane column with a mobile phase consisting of 0.01 M sodium acetate: acetonitrile (4:1 v/v) at a flow rate of 1.6 mL/min. A detection limit of 30 ng/mL was accomplished at 226 nm.

A review of the procedures for the determination of Chlorthalidone in biological fluids is given by Johnston et.al. (23).

## 7. BIOAVAILABILITY AND PHARMACOKINETICS

The pharmacology of Chlorthalidone has been studied extensively. Initial animal experiments indicated that the pharmacological effects were limited to diuresis and saluresis (18, 24, 25). From these early experiments, the investigators concluded that Chlorthalidone acts on the renal tubuli through an inhibition of sodium reabsorption while only nominally affecting potassium excretion. Ford (26) monitored ten patients with hypertension who were maintained on a 50 mEq diet of sodium. Over a 24 hour period, urinary excretion of sodium increased 112 mEq/24 hrs. after a single 25 mg dose and increased 164 mEq/24 hrs. after a single 50 mg dose. Potassium excretion increased only minimally with respect to the controls at the 25 and 50 mg doses. Chlorthalidone has been reported to be well tolerated and with minimal side effects (27).

The work of Dorsey et. al. (28) demonstrated the bioavailability of Chlorthalidone. The results of a study on 7 healthy subjects showed the mean relative bioavailability of a 25 mg tablet dose to be  $110.3 \pm 8.1\%$  compared with an orally-administered solution. Chlorthalidone is rapidly absorbed in humans, as demonstrated by half-life values of 0.44 and 0.25 hr. for the tablet and solution, respectively (28). The time to peak urinary excretion rate for the tablet dosage form was determined to be 2.1 hr. as compared to 1.3 hr. for the solution. These results are in agreement with earlier values for peak plasma concentrations.

The drug displays a relatively long and variable elimination half-life and has been determined by several authors to be within the range of 25-80 hrs. (19, 28-33), with a mean half-life of about 50 hrs. (28, 31).

Chlorthalidone has been found to bind strongly to the red blood cells (14, 17, 29, 31, 34, 35). At equilibrium, between 94-99% of the amount of Chlorthalidone in the blood is bound to the erythrocytes (34, 35). The receptors responsible for the binding of Chlorthalidone to human red blood cells have been identified as two major isoenzymes of carbonic anhydrase (31, 35). In human serum, Chlorthalidone is mostly associated with the albumin fraction, although the binding is considerably weaker (35). It has been found that equilibration of the drug between the plasma and red blood cell fractions occurs 10-12 hrs. after administration (14). These factors appear to account significantly for the long biologic half-life of the drug (32, 36).

Investigators have reported that between 30-60% of the daily dose of Chlorthalidone is excreted as unchanged drug (19, 31). The remaining fraction of the drug apparently is eliminated via the liver, either through metabolism or fecal excretion (32, 37). However, biliary excretion of unchanged drug does not appear to be the major pathway since only 0.6 - 1.4% of the administered dose has been found in patient studies by Fleuren et. al. (37). The difference appears to be due to metabolic degradation of the drug. Beisenherz et. al. (17) identified the 4-chloro-3-sulfamylbenzophenone-2-carboxylic acid (CCA) as the only metabolic product in rats administered with <sup>14</sup>C-Chlorthalidone. Unidentified polar material was detected by Beerman et. al (34) in human urine. However, more recent studies (37) have not been able to detect CCA in either human urine or bile. The actual metabolic pathway of Chlorthalidone is still subject to speculation.

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# IMIPRAMINE HYDROCHLORIDE

Donald N. Kender and  
Richard E. Schiesswohl

*CIBA-GEIGY Corporation  
Suffern, New York*

1. History and Therapeutic Category	38
2. Description	38
2.1 Chemical Names	38
2.2 Trade Names	38
2.3 Research Number	38
2.4 Chemical Abstracts Registry Number	38
2.5 Chemical Structure, Molecular Formula, and Molecular Weight	38
2.6 Appearance, Odor, and Color	39
3. Synthesis	39
4. Physical Properties	39
4.1 Infrared Spectroscopy	39
4.2 Proton Nuclear Magnetic Resonance Spectroscopy	41
4.3 Carbon-13 Nuclear Magnetic Resonance Spectroscopy	41
4.4 Ultraviolet Absorption Spectroscopy	44
4.5 Mass Spectroscopy	46
4.6 Melting Range	48
4.7 Differential Scanning Calorimetry	48
4.8 Thermogravimetric Analysis	48
4.9 Solubility	48
4.10 Distribution Ratio	50
4.11 Dissociation Constant	50
4.12 X-Ray Analysis	50
5. Methods of Analysis	52
5.1 Elemental Analysis	52
5.2 Ultraviolet Absorption Spectroscopy	52
5.3 Colorimetric Methods	52
5.4 Titration	53
5.5 Atomic Absorption	53
5.6 Polarography	53
5.7 Chromatography	53
6. Stability–Degradation	58
7. Metabolic Products–Pharmacokinetics	64
8. Identification and Determination in Body Fluids and Tissues	64
9. Identification and Determination in Pharmaceuticals	65
References	72

## 1. History and Therapeutic Category

Imipramine hydrochloride, the original tricyclic antidepressant, is a member of the dibenzazepine group of compounds. It was originally synthesized (1) in the 1950's by J. R. Geigy, Ltd. in Basle, Switzerland as an outgrowth of their antihistamine development program. It was through the clinical observations of Kuhn that the antidepressant properties of imipramine hydrochloride were discovered, and these findings were first published in 1957 (2). By 1970 there were over 5000 publications on the new drug substance (3).

Imipramine hydrochloride is approved for depression and childhood enuresis in the US (4). Additional indications for which clinical data exist have been reviewed by Angst, et.al. (5). Imipramine is also commercially available as a pamoate salt (4). Additional historical information on the discovery of imipramine hydrochloride has been outlined by Kuhn (3).

## 2. Description

### 2.1 Chemical Names

- i. 5-[3-(Dimethylamino)propyl]-10,11-dihydro-5H-dibenz[b,f]azepine Monohydrochloride
- ii. 10,11-Dihydro-N,N-dimethyl-5H-dibenz[b,f]-azepine-5-propanamine Monohydrochloride
- iii. N-( $\gamma$ -Dimethylaminopropyl)iminodibenzyl Monohydrochloride

### 2.2 Trade Names

Tofranil, SK-Pramine, Janimine

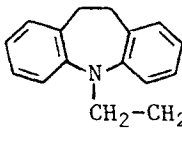
### 2.3 Research Number

G 22355 (CIBA-GEIGY)

### 2.4 Chemical Abstracts Registry Number

[113-52-0]

### 2.5 Chemical Structure, Molecular Formula and Molecular Weight



• HCl

C<sub>19</sub>H<sub>24</sub>N<sub>2</sub>•HCl

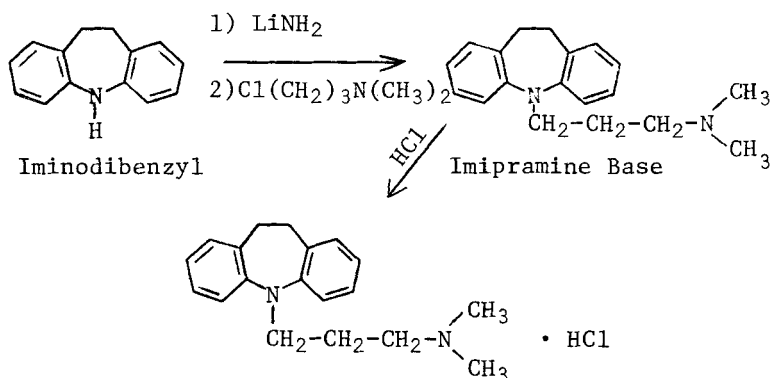
M.W. 316.87 (free base 280.42)

## 2.6 Appearance, Odor and Color

Imipramine hydrochloride is a white to off-white, odorless or practically odorless, crystalline powder.

## 3. Synthesis

The synthesis of imipramine hydrochloride is outlined below (1,6).



## Imipramine Hydrochloride

## 4. Physical Properties

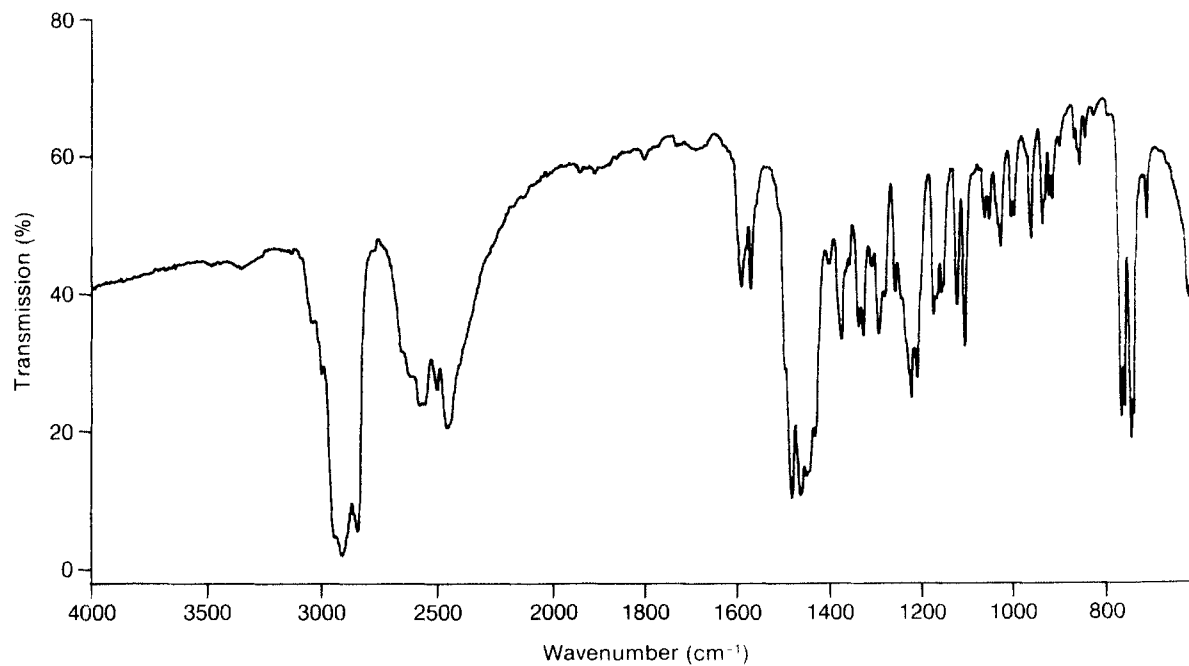
### 4.1 Infrared Spectroscopy

The infrared absorption spectrum of imipramine hydrochloride obtained as a Nujol mull on a Perkin-Elmer Model 281B spectrophotometer is given in Figure 1. Assignments for the major absorption bands are given in Table I.

TABLE I  
Infrared Absorption Assignments

Wavenumber ( $\text{cm}^{-1}$ )	Assignment(s)
3000-2900, 1460-1440	Aliphatic CH and Nujol
2570, 2470	N•HCl
1600, 1575, 1490	Aromatic CH
775, 750	

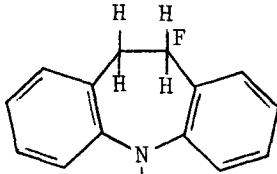
Figure 1.  
Infrared Absorption Spectrum of Imipramine Hydrochloride Obtained as a Nujol Mull.



#### 4.2 Proton Nuclear Magnetic Resonance Spectroscopy

The 90 MHz proton nuclear magnetic resonance (NMR) spectrum of imipramine hydrochloride obtained in  $\text{CDCl}_3$  is given in Figure 2. The spectrum has been obtained on a JEOL FX90Q NMR instrument. The chemical shifts and assignments are given in Table II. These data are consistent with data previously reported for imipramine hydrochloride and the free base (7,8). From analysis of the proton NMR data it can be shown that the imipramine hydrochloride propyl side chain has a preferred conformation of gauche  $\text{C}_\text{A}-\text{C}_\text{B}$  and trans  $\text{C}_\text{B}-\text{C}_\text{C}$  in solution (7).

TABLE II  
Proton NMR Assignments

 <p style="text-align: center;"> <math>\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{N}(\text{CH}_3)_2 \cdot \text{HCl}</math>  A      B      C                  D                  E </p>			
Position	Chemical Shift $\delta$ (ppm)	Number of Protons	Multiplicity
E	12.2	1	Broad
Aromatics	7.3-6.8	8	Multiplet
A	3.8	2	Triplet
F	3.1	4	Singlet
C	3.0	2	Triplet
D	2.6	6	Doublet
B	2.1	2	Multiplet

#### 4.3 Carbon-13 Nuclear Magnetic Resonance Spectroscopy

The  $^{13}\text{C}$  NMR spectrum of imipramine hydrochloride obtained in  $\text{CDCl}_3$  is given in Figure 3. The spectrum has been obtained on a JEOL FX90Q NMR instrument at 22.5 MHz. The chemical shifts and assignments are given in Table III. These data are consistent with data reported in the literature (7).



Figure 2.  
Proton Nuclear Magnetic Resonance Spectrum of Imipramine Hydrochloride in  $\text{CDCl}_3$

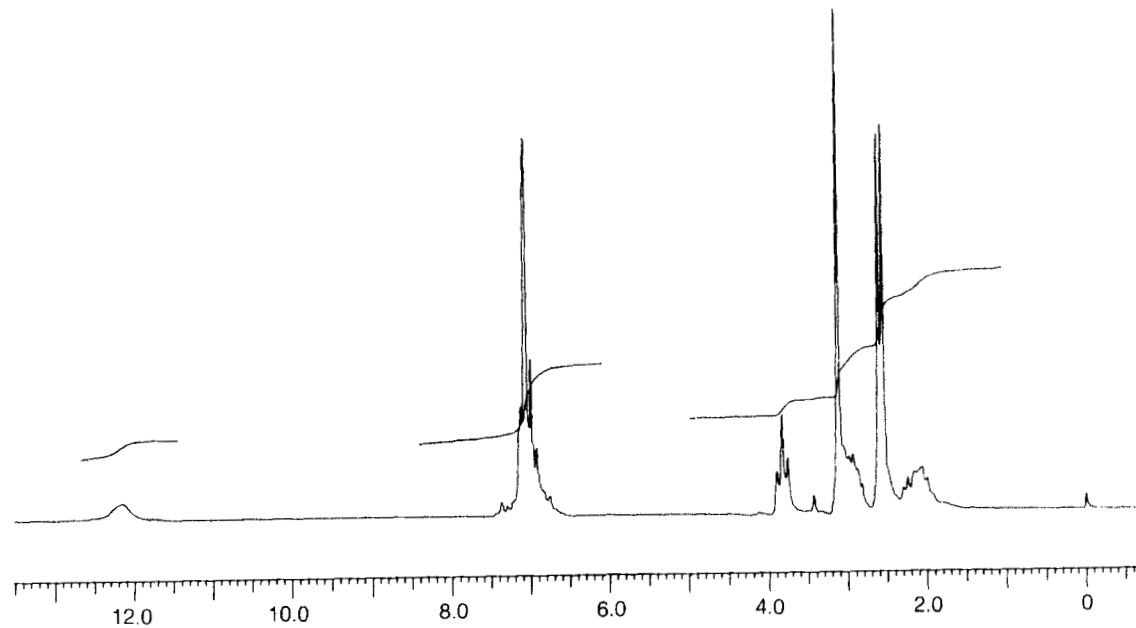


Figure 3.  
Carbon-13 Nuclear Magnetic Resonance Spectrum of Imipramine Hydrochloride in  $\text{CDCl}_3$ .

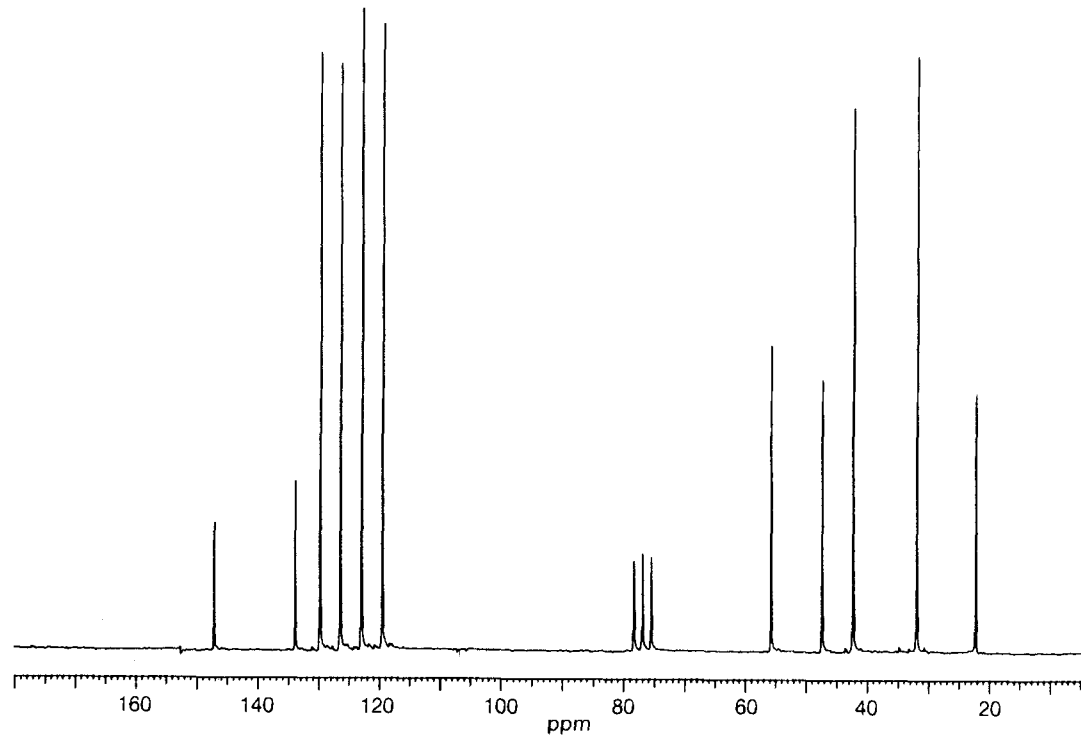
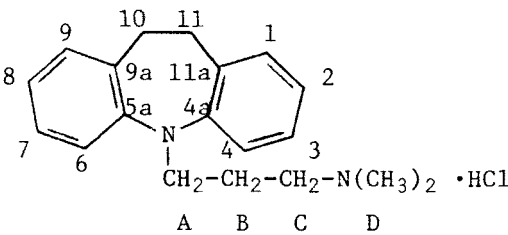


TABLE III  
Carbon-13 NMR Assignments

		
Position	Chemical Shift $\delta_{\text{TMS}}$ (ppm)	Multiplicity Without $^1\text{H}$ -Decoupling
1,9	129.9	Doublet
2,8	119.6	Doublet
3,7	126.5	Doublet
4,6	123.0	Doublet
10,11	32.0	Triplet
9a,11a	134.0	Singlet
4a,5a	147.3	Singlet
A	47.4	Triplet
B	22.3	Triplet
C	55.8	Triplet
D	42.4	Quartet

#### 4.4 Ultraviolet Absorption Spectroscopy

The ultraviolet absorption spectrum of imipramine hydrochloride in 0.1N hydrochloric acid is given in Figure 4. The spectrum has been determined on a Hewlett-Packard Model 8450A spectrophotometer. The wavelength maxima ( $\lambda_{\text{max}}$ ) and molar absorptivities ( $\epsilon$ ) for imipramine hydrochloride in several solvents are given in Table IV.

Figure 4.  
Ultraviolet Absorption Spectrum of Imipramine  
Hydrochloride in 0.1N Hydrochloric Acid.

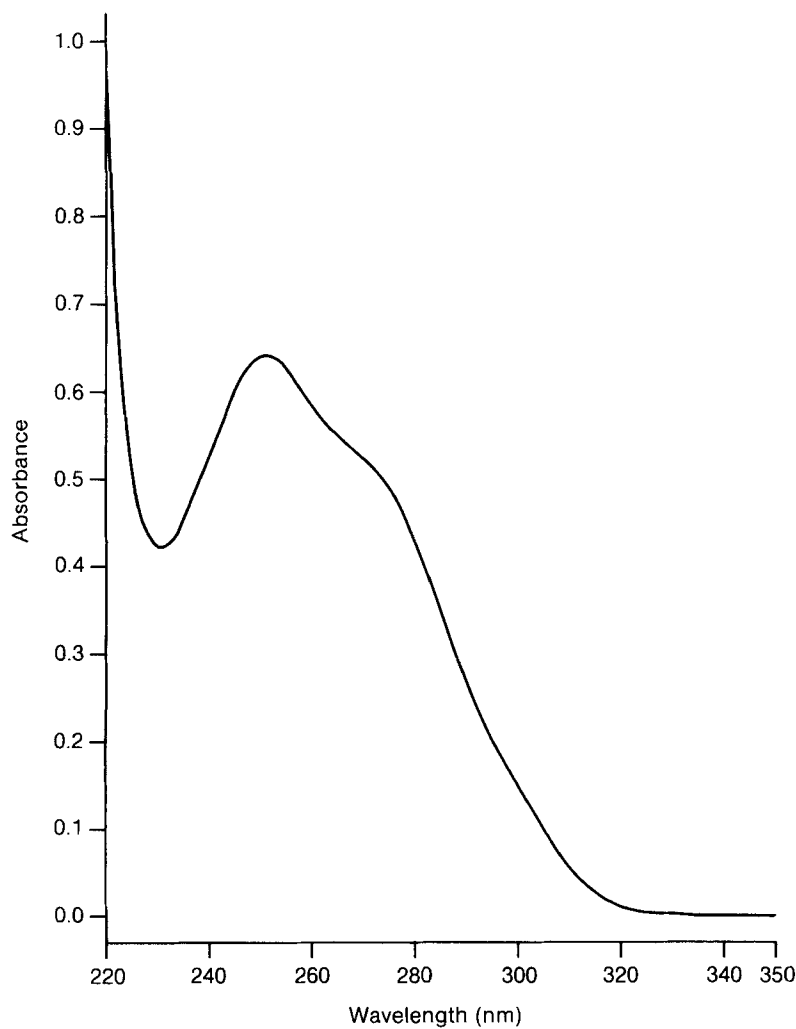


TABLE IV  
Ultraviolet Absorption Data

Solvent	$\lambda_{\text{max}}$ (nm)	$\epsilon \times 10^{-3}$	Reference
0.1N HCl	251	8.40	9
0.01N HCl	251	8.40	9
Aqueous pH <7	251	8.41	10
1-Pentanol/ Cyclohexane (2:8)	255	9.54	10

#### 4.5 Mass Spectroscopy

Presented in Figure 5 is the 70 eV electron impact mass spectrum of imipramine hydrochloride obtained on a Kratos MS 25 mass spectrometer using a solid probe. The mass spectrum of imipramine hydrochloride is the spectrum of its free base resulting from thermal dissociation when the compound is vaporized. An interpretation of the spectrum is given in Table V. These data are consistent with literature values (8,11,12). Chemical ionization with methane yields predominantly the  $\text{MH}^+$  at  $m/z=281$  and minor peaks at  $m/z=208$ , 309 and 321 (13).

TABLE V  
Mass Spectral Assignments

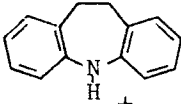
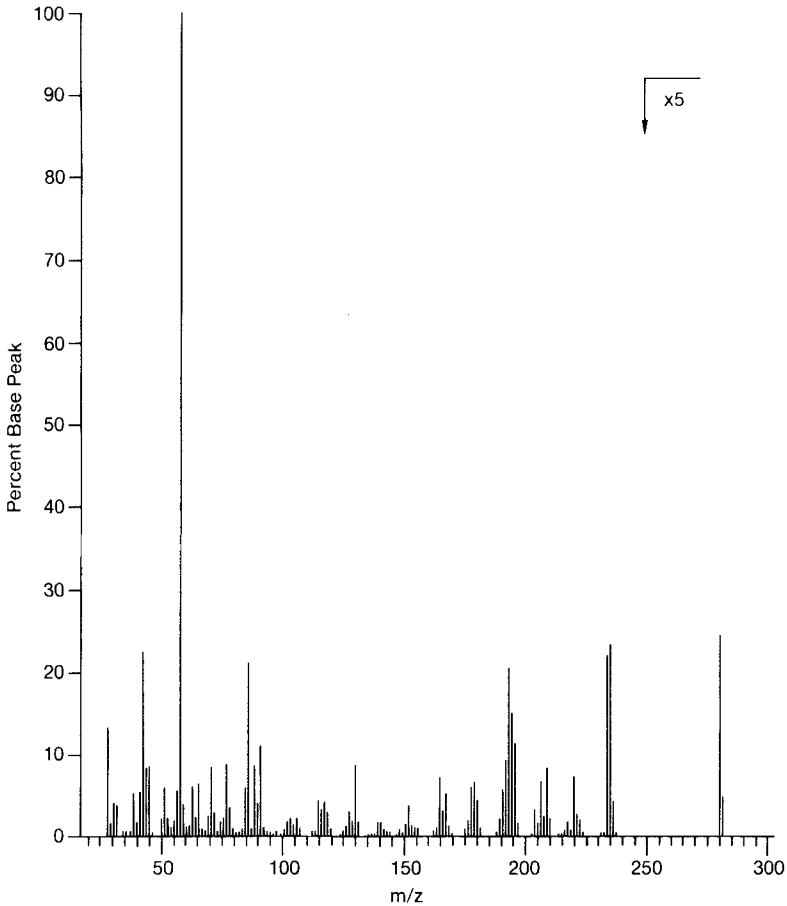
$m/z$	Assignment
280	$\text{M}^+$ (free base)
235	$\text{M}^+ - (\text{CH}_3)_2\text{NH}$
220	$\text{M}^+ - (\text{CH}_3)_2\text{NCH}_2 - \text{H}_2$
208	$\text{M}^+ - (\text{CH}_3)_2\text{NCH}_2\text{CH}_2$
195	
85	$\text{CH}_2\text{CH}_2\text{CH}=\text{N}^+(\text{CH}_3)_2$
58	$(\text{CH}_3)_2\text{N}^+=\text{CH}_2$

Figure 5.  
Electron Impact Mass Spectrum of Imipramine Hydrochloride.



#### 4.6 Melting Range

Imipramine hydrochloride melts over a 1-2 degree range between 170-174°C (9,14). A value of 174°-175° (acetone) has been reported (1). The free base has a boiling point of 160° at 0.1 mm (1).

#### 4.7 Differential Scanning Calorimetry

The differential scanning calorimetry curve of imipramine hydrochloride, obtained on a Perkin-Elmer DSC 2 instrument at a scan rate of 1.25°K/min, exhibits an endotherm with a peak temperature at 446.6°K as shown in Figure 6. A heat of fusion value of 7702.8 cal/mole has been obtained for an imipramine hydrochloride sample having a purity of 99.8 mole percent.

#### 4.8 Thermogravimetric Analysis

Thermogravimetric analysis of imipramine hydrochloride typically exhibits a weight loss of less than 0.1% between room temperature and 150°C. Sublimation occurs at about 160°C (9).

#### 4.9 Solubility

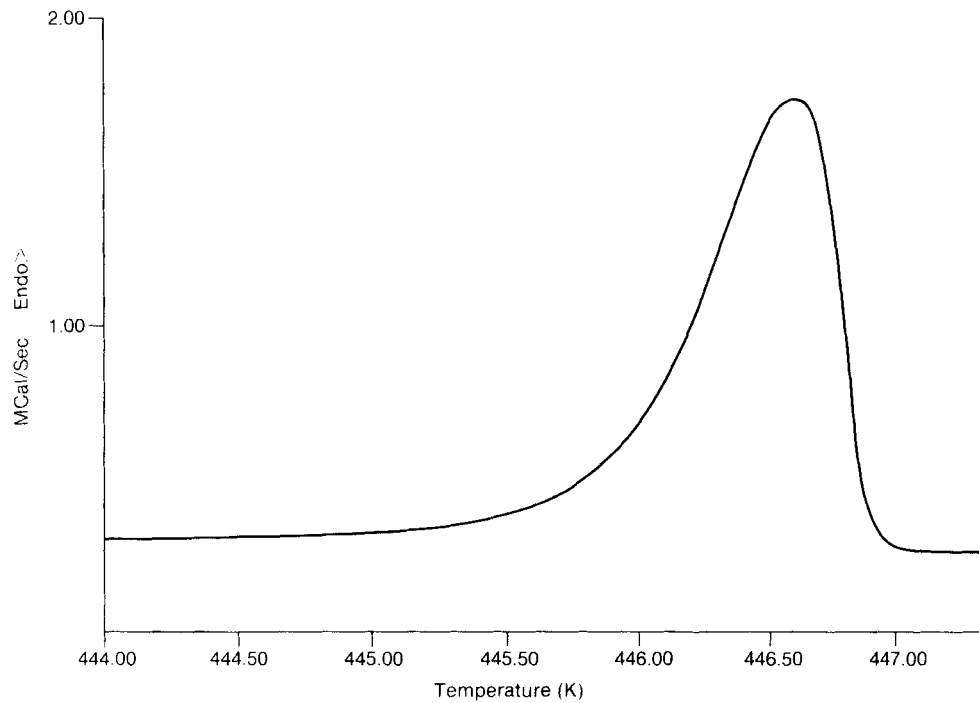
The following solubilities given in Table VI have been determined for imipramine hydrochloride at room temperature (9).

TABLE VI  
Solubility Data

Solvent	g/100 ml
Water (pH 4.55)	50
Simulated Gastric Fluid	10
Ethanol	73
Chloroform	20
Ether	0.01

In addition, imipramine base has been reported to have a solubility of 1.82 mg/100 ml and 0.42 mg/100 ml in water and pH 7.4 buffer, respectively (15).

Figure 6.  
Differential Scanning Calorimetry Curve of Imipramine Hydrochloride.





#### 4.10 Distribution Ratio

Distribution ratio data on imipramine hydrochloride, expressed as the organic phase concentration divided by the aqueous phase concentration, are summarized in Table VII.

TABLE VII  
Distribution Ratios

Organic Phase	Aqueous Phase	Temperature (°C)	$P = C_{\text{org}}/C_{\text{aq}}$	Reference
Chloroform		20	499	9
		24	>500	16
n-Hexane	Isotonic Phosphate Buffer pH 7.4	20	165	9
		24	166	16
Ether		20	142	9

#### 4.11 Dissociation Constant

Dissociation constant data (pKa) for imipramine hydrochloride are listed in Table VIII.

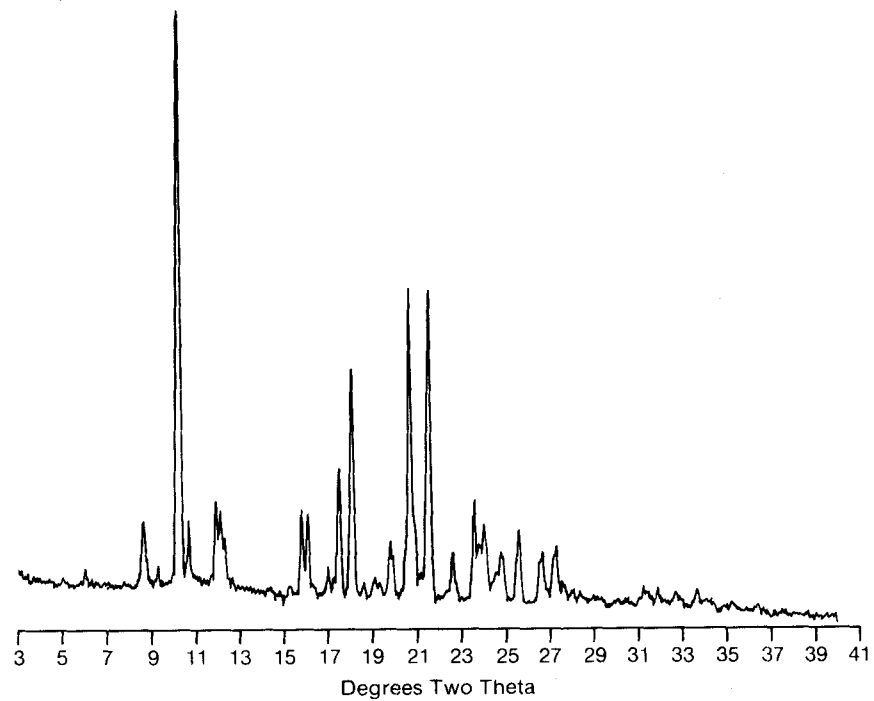
TABLE VIII  
Dissociation Constants

Temperature (°C)	Method	pKa	Reference
22	Photometric Titration	9.62	9
24	Solubility	9.5	9,15
--	Extrapolated from Water/Methylcellosolve Mixture; Potentiometric Titration	9.5	9

#### 4.12 X-ray Analysis

The X-ray powder diffraction pattern obtained for imipramine hydrochloride on a Diano Model 8535 Diffractometer using the  $\text{CuK}_\alpha$  line (1.542Å) as the radiation source with a Ni filter is shown in Figure 7. Imipramine hydrochloride has also been subjected to single crystal X-ray analysis in order to establish the conformational properties

Figure 7.  
X-ray Powder Diffraction Pattern of Imipramine Hydrochloride.



of the dibenzazepine ring system and the dimethylaminopropyl side chain (17). Analysis of the crystalline data shows that the angle of annellation between the b and f bonds is 40°; the angle of flexure between the planes of the two benzene rings is 55°; the angle of torsion, which represents the extent to which the molecule is twisted out of a symmetrical conformation, is 20°; and the distance between the center of the benzene rings is 5.1Å (18).

## 5. Methods of Analysis

### 5.1 Elemental Analysis

The elemental composition for a typical sample of imipramine hydrochloride obtained on a Perkin-Elmer Model 240 CHN Analyzer is given below. These data are consistent with previous findings (1).

Elements	Theory (%)	Found (%)
C	72.02	71.86
H	7.95	7.67
O	8.84	8.60

### 5.2 Ultraviolet Absorption Spectroscopy

Ultraviolet absorption spectroscopy (See Section 4.4) can be used for the quantitation and identification of imipramine hydrochloride itself and in dosage forms (14,19). Automated methods of analysis have been developed for imipramine hydrochloride tablets which have been shown to be accurate and precise (20,21).

### 5.3 Colorimetric Methods

Imipramine hydrochloride can be determined in pharmaceutical dosage forms using the methods outlined in Table IX. In addition, color tests have been used to detect and identify imipramine hydrochloride. These have included the Keller Test (22), Marquis Reagent (22), nitric acid (22), and cis-aconitic anhydride (23).

TABLE IX  
Colorimetric Methods of Analysis

Reagent	$\lambda_{\text{max}}$ (nm)	Reference
Nitrous Acid in Dilute Hydrochloric Acid	390	19
Bromothymol Blue Extraction into Benzene	410	19
Potassium Hexaiodobismutate Extraction into Chloroform	490	24
Tetrathiocyanodiamine Chromate in Acetone	540	25

#### 5.4 Titration

Imipramine hydrochloride can be titrated in glacial acetic acid with 0.1N perchloric acid using crystal violet TS as an indicator (14). Imipramine hydrochloride may also be determined by titration of excess silver nitrate with potassium iodide using a Cu(II) salt as an indicator (26) or by the reaction of imipramine hydrochloride with potassium hexathiocyanatochromate and bromatometric titration of the excess reagent (27).

#### 5.5 Atomic Absorption

Imipramine hydrochloride can be quantitated by reaction with sodium dioctylsulfosuccinate. The excess sodium dioctylsulfosuccinate is determined after reaction with cupric ortho-phenanthroline and extraction with methylisobutylketone. Copper is assayed in the organic phase by atomic absorption (28).

#### 5.6 Polarography

Polarography can be used to determine the imipramine hydrochloride content of dosage forms in the pH range of 6.5-8.0 (29).

#### 5.7 Chromatography

A multitude of gas, liquid and thin layer chromatographic methods have been developed for imipramine hydrochloride. In this Section the

TABLE X

Thin Layer Chromatographic Methods

Support	Solvent System	Detection	rf	Reference
Silica Gel	Acetic Acid + Ethyl Acetate + Water + HCl (35:55:5:5)	1. UV @ 366nm 2. $K_2Cr_2O_7$ in 20% $H_2SO_4$ 3. UV @ 366nm after 2 hrs.	0.27	30
	Benzene + Ethyl Acetate + Ethanol + $NH_4OH$ (50:50:10:5 upper phase)	"	0.63	30
	Benzene + Ethyl Acetate + Ethanol + $NH_4OH$ (50:50:15:3)	1. Fluorescence Quenching 2. $K_2Cr_2O_7$ in 20% $H_2SO_4$	0.67	31
	Benzene + Acetone + $NH_4OH$ (300:60:1)	Fluorescence Quenching	0.24	8
	Benzene + Dioxane + Methanol + $NH_4OH$ (100:80:10:1)	"	0.60	8
	Methanol + $NH_4OH$ (200:3)	"	0.62	8

TABLE X Continued

Silica Gel	Acetone + NH <sub>4</sub> OH (100:1)	Fluorescence Quenching	0.65	8
	Chloroform + Methanol + NH <sub>4</sub> OH (50:50:1)	"	0.75	8
	Dioxane + Chloroform + Acetone + NH <sub>4</sub> OH (95:90:10:5)	"	0.76	8
	Benzene + Ethyl Acetate + Methanol + NH <sub>4</sub> OH (75:75:15:2)	"	0.84	8
	Benzene + Methanol + NH <sub>4</sub> OH (133:21:2)	"	0.89	8
	Chloroform + Methanol (1:1)	"	0.33	8
	Chloroform + Methanol (4:1)	"	0.54	8
	Chloroform + Methanol + Water (4:2:1) [Lower phase, plus 5% Methanol]	"	0.69	8
	Benzene + Methanol + Acetic Acid (50:50:1)	"	0.32	8
	Chloroform + Methanol + Acetic Acid (50:50:1)	"	0.33	8

TABLE X Continued

Silica Gel	Ethyl Acetate + Methanol + Acetic Acid (45:255:2)	Fluorescence Quenching	0.33	8
	Methanol + Acetic Acid (150:1)	"	0.38	8
	Methanol + NH <sub>4</sub> OH (100:1.5)	1. Fluorescence Quenching 2. Iodoplatinate	0.50	32
	Cyclohexane + Diethylamine (8:1)	"	0.86	32
	Cyclohexane + Ethanol + Butanol + NH <sub>4</sub> OH (80:20:10:0.4)	Fluorescence Quenching	0.47	33
Woelm Alumina (Basic)	Methanol	Fluorescence Quenching	0.69	8
	Chloroform + Isopropanol (1:1)	"	0.87	8
Avicel Micro- crystalline Cellulose	Methanol + Water + NH <sub>4</sub> OH (100:45:1)	Fluorescence Quenching	0.92	8
	Methanol + Water + Acetic Acid (60:90:1)	"	0.85	8

chromatography is limited to separation of synthetic and degradation by-products from imipramine or chromatography of the active ingredient itself and dosage forms. Other chromatographic techniques used for imipramine hydrochloride are outlined in Section 8.

#### 5.7.1 Thin Layer Chromatography

The classical paper on the thin layer chromatography of imipramine hydrochloride was published by Adank and Hammerschmidt in 1964 (30). Using two solvent systems they were able to separate all the major synthetic and degradation by-products (See Section 6). Various thin layer chromatographic methods used for imipramine hydrochloride are outlined in Table X.

#### 5.7.2 Gas Chromatography

Gas chromatography has been used for the determination of imipramine hydrochloride in dosage forms and for the quantitation of by-products. These methods are outlined in Table XI.

TABLE XI  
Gas Chromatographic Methods

Packing Material	Column Temperature (°C)	Detector	Reference
-, 5% OV-25 on Chromosorb W-HP (100-120 mesh)	240	FID	31
6' x 1/4", 5% OV-17 on Gas Chrom Q (80-100 mesh)	230	FID	34
6' x 4 mm, 10% Dexsil 300 on Chromosorb W-HP (80-100 mesh)	258	FID	35
0.9 m x 2 mm, 3% SP 2250 on Supelcoport (80-100 mesh)	T <sub>i</sub> =200° for 1 min.; 10°/min. to T <sub>f</sub> =250°	MS	36
6' x 2 mm, 3% OV-17 on Gas Chrom Q (100-120 mesh)	190, 240	FID	37



### 5.7.3 High Pressure Liquid Chromatography

High pressure liquid chromatography has been used for the determination of imipramine hydrochloride in dosage forms and for the quantitation of by-products. These methods are outlined in Table XII.

TABLE XII  
HPLC Methods

Column	Mobile Phase	Detector	Reference
Lichrosorb Si 60	Cyclohexane + Ethanol + Butanol + NH <sub>4</sub> OH (80:20:10:0.4)	UV <sub>254</sub>	33
μBondapak-C <sub>18</sub>	Acetonitrile + Water (40:60 or 55:45) containing 0.005M Heptanesulfonic Acid and 1% Acetic Acid	UV <sub>254</sub>	38
Zorbax-SIL	Methylene Chloride + Methanol + Water + Diethylamine (850:150:1.0:0.25)	UV <sub>251</sub>	39

## 6. Stability-Degradation

Imipramine hydrochloride is stable as a solid and in aqueous solution. The degradation of imipramine hydrochloride under accelerated conditions has been extensively studied by Adank and Hammerschmidt (30), and the pathway of degradation is presented in Figure 8. The degradation of imipramine hydrochloride is similar to the degradation of other related dibenzazepine compounds (40). In aqueous solutions at pH 4, iminodibenzyl is the major by-product produced in heated samples (41). Iminodibenzyl can also be produced as an unwanted by-product during derivatization procedures for gas chromatography (42). In addition, studies on the degradation of imipramine-N-oxide (39) and the mechanism of oxidation of imipramine hydrochloride to cation radicals by means of Ce(IV) in H<sub>2</sub>SO<sub>4</sub> (43) have been published.

Figure 8.  
Degradation Pathway of Imipramine Hydrochloride (30).

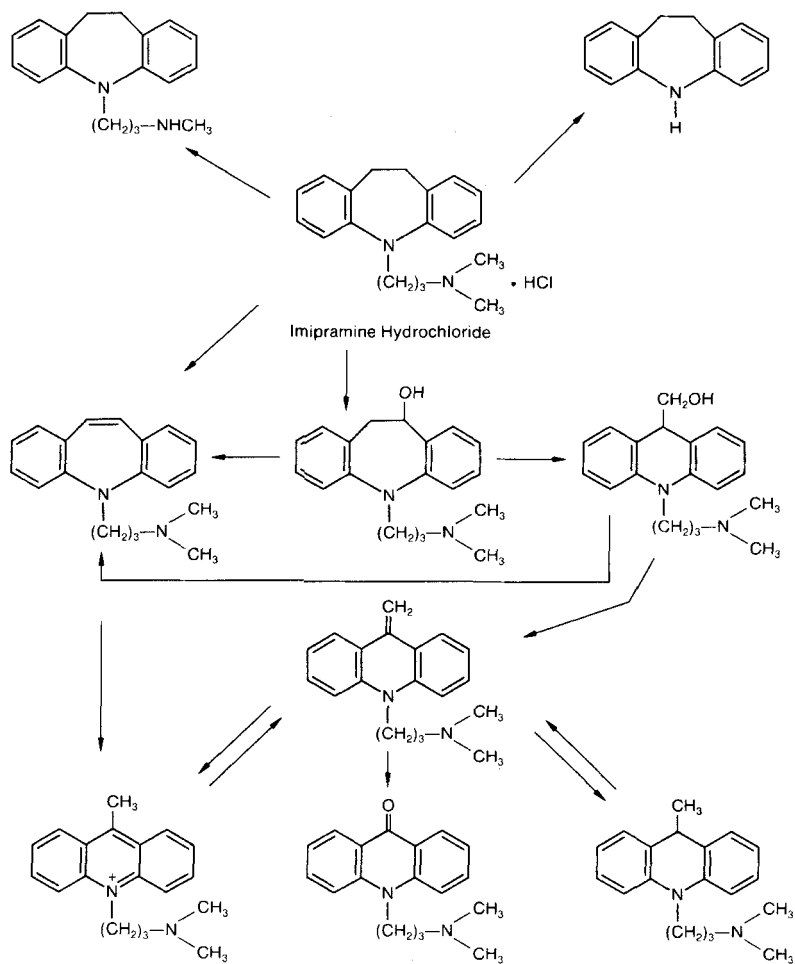
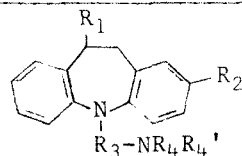


TABLE XIII

## Metabolic Products



Abbreviation	Description	R <sub>1</sub>	R <sub>2</sub>	R <sub>3</sub>	R <sub>4</sub>	R <sub>4</sub> '
IP	Imipramine	H	H	(CH <sub>2</sub> ) <sub>3</sub>	CH <sub>3</sub>	CH <sub>3</sub>
DMI	Desmethylimipramine	H	H	(CH <sub>2</sub> ) <sub>3</sub>	CH <sub>3</sub>	H
DDMI	Desdesmethylimipramine	H	H	(CH <sub>2</sub> ) <sub>3</sub>	H	H
IPNO	Imipramine-N-oxide	H	H	(CH <sub>2</sub> ) <sub>3</sub>	CH <sub>3</sub>	CH <sub>3</sub> (N→O)
IDB	Iminodibenzyl	H	H	H	-	-
2-OH-IP	2-Hydroxyimipramine	H	OH	(CH <sub>2</sub> ) <sub>3</sub>	CH <sub>3</sub>	CH <sub>3</sub>
2-OH-DMI	2-Hydroxydesmethylimipramine	H	OH	(CH <sub>2</sub> ) <sub>3</sub>	CH <sub>3</sub>	H
2-OH-DDMI	2-Hydroxydesdesmethylimipramine	H	OH	(CH <sub>2</sub> ) <sub>3</sub>	H	H
2-OH-IDB	2-Hydroxyiminodibenzyl	H	OH	H	-	-
10-OH-IP	10-Hydroxyimipramine	OH	H	(CH <sub>2</sub> ) <sub>3</sub>	CH <sub>3</sub>	CH <sub>3</sub>
10-OH-DMI	10-Hydroxydesmethylimipramine	OH	H	(CH <sub>2</sub> ) <sub>3</sub>	CH <sub>3</sub>	H
10-OH-DDMI	10-Hydroxydesdesmethylimipramine	OH	H	(CH <sub>2</sub> ) <sub>3</sub>	H	H
2-OH-IP-GA†	2-Hydroxyimipramine glucuronide	H	O-GA	(CH <sub>2</sub> ) <sub>3</sub>	CH <sub>3</sub>	CH <sub>3</sub>
2-OH-DMI-GA	2-Hydroxydesmethylimipramine glucuronide	H	O-GA	(CH <sub>2</sub> ) <sub>3</sub>	CH <sub>3</sub>	H
2-OH-IDB-GA	2-Hydroxyiminodibenzyl glucuronide	H	O-GA	H	-	-

TABLE XIII Continued

10-OH-IP-GA	10-Hydroxyimipramine glucuronide	O-GA	H	(CH <sub>2</sub> ) <sub>3</sub>	CH <sub>3</sub>	CH <sub>3</sub>
10-OH-DMI-GA	10-Hydroxydesmethylimipramine glucuronide	O-GA	H	(CH <sub>2</sub> ) <sub>3</sub>	CH <sub>3</sub>	H
10-OH-DDMI-GA	10-Hydroxydesdesmethylimipramine glucuronide	O-GA	H	(CH <sub>2</sub> ) <sub>3</sub>	H	H
†Glucuronic Acid						

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Figure 9.  
Chemical Scheme for the Metabolism of  
Imipramine Hydrochloride (56).

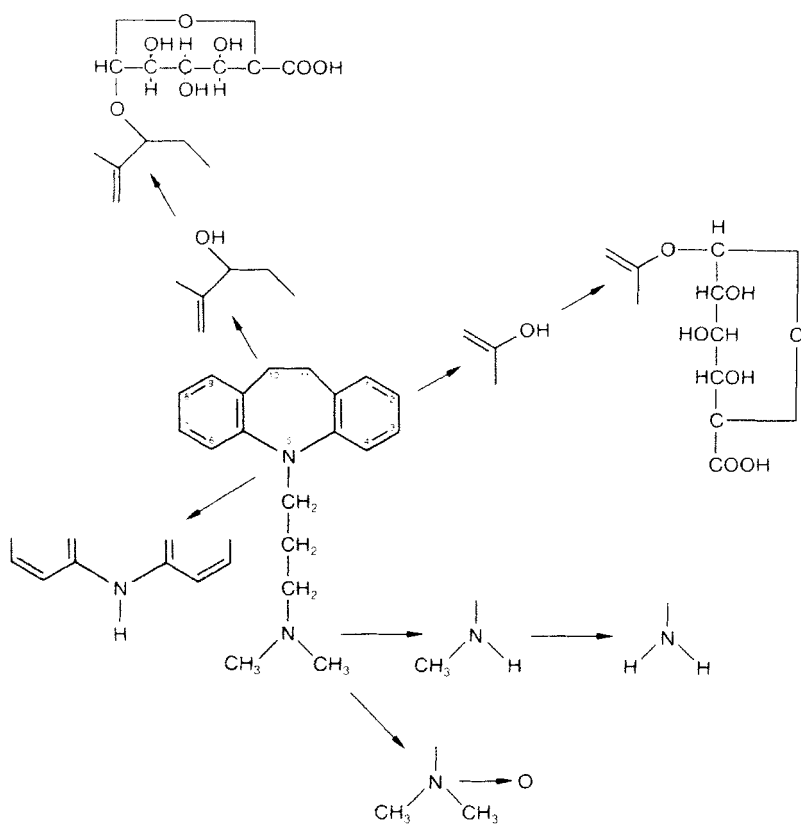
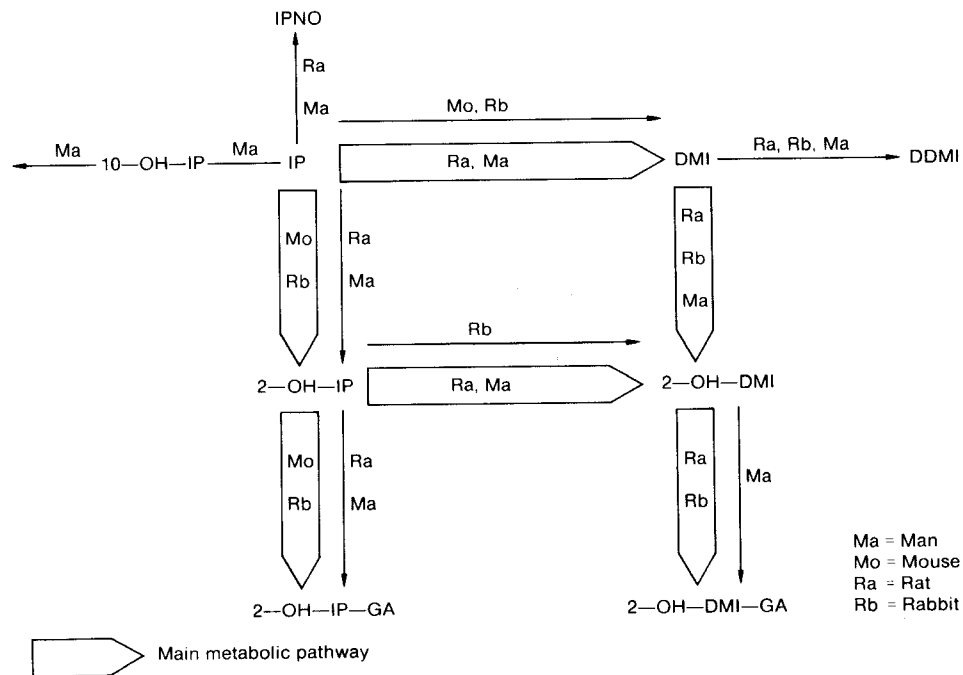


Figure 10.  
Metabolic Pathway of Imipramine Hydrochloride in Man, Mouse, Rabbit, and Rat (57).



## 7. Metabolic Products-Pharmacokinetics

Imipramine hydrochloride is well absorbed when given orally as demonstrated by blood concentrations and excretion of the drug after oral and after intravenous administration (44,45). The absolute bioavailability of an oral dose ranges from about 30 to 77 percent (45-47), and studies on the relative bioavailability of different solid and liquid oral formulations have been performed (48,49). The variable absolute bioavailability is the result of rapid first pass metabolism (45,47,50). The plasma half-life has been reported to be about 6-25 hours (47,51-53), and protein binding is reported to be on the order of 56-96 percent (47,54). In addition, pharmacokinetic properties have been studied versus age and sex (52) and in the elderly (55).

The metabolism of imipramine hydrochloride has been extensively studied, and excellent reviews are available (56,57). The structures and abbreviations for the metabolic products are given in Table XIII (57), and the chemical scheme for the metabolism is given in Figure 9 (56). A schematic showing the pathway of metabolism is given in Figure 10 for the man, mouse, rabbit and rat (57). In addition, the metabolism has also been studied in microorganisms (58).

Imipramine hydrochloride has been shown to be substantially metabolized in the liver, to a very small percent in the kidney and in the lung, and essentially not metabolized by other extrahepatic tissues (50,59). In humans the metabolism to desipramine has also been shown to occur in the liver (60).

Quantitative determinations of imipramine and its metabolites have been determined in plasma (46,52,61). In addition, quantitative determinations of excreted imipramine and its metabolites have been determined in urine and feces (44,45) and human breast milk (62).

## 8. Identification and Determination in Body Fluids and Tissues

A general review on methodology to determine tricyclic antidepressants in body fluids and tissues, including imipramine hydrochloride, has been published by Scoggins, et. al. (63). Although imipramine hydrochloride can be determined directly by such techniques

as ultraviolet/visible spectroscopy (64) and fluorescence (65), most methods rely on chromatographic treatment. These methods are outlined in Table XIV for thin layer chromatography, Table XV for high pressure liquid chromatography, and Table XVI for gas chromatography which includes gas chromatography-mass spectroscopy.

9. Identification and Determination in Pharmaceuticals

The following tabulation highlights the methods used in the identification and determination of imipramine hydrochloride in pharmaceutical dosage forms.

Method	Reference
Colorimetric	19, 24
Gas Chromatography	31, 34, 35, 36, 37
High Pressure Liquid Chromatography	38
Infrared Spectroscopy	14
Polarography	29
Thin Layer Chromatography	30, 31, 37
Titration	26, 27
Ultraviolet Spectroscopy	14, 19, 20, 21

Acknowledgement

The authors express their appreciation to Jane Thompson and Susan Case for their help in preparing this manuscript.



TABLE XIV

Thin Layer Chromatographic Methods

Support	Solvent System	Detection	Source*	Reference
Silica Gel	Ethyl Acetate + Methanol + NH <sub>4</sub> OH (81:15:4)	Colorimetric	M	58
	Acetone + NH <sub>4</sub> OH (40:1)	Colorimetric	B	65
	Chloroform + Toluene + Diethylamine (11:6:3)	Colorimetric	B	65
	Chloroform + Toluene + Methanol + NH <sub>4</sub> OH (4:3:2:1 lower phase)	Colorimetric	B	65
	n-Propanol + NH <sub>4</sub> OH + Water (16:1:3)	Colorimetric	B	65
	Chloroform + Ethyl Ether + Methanol (85:15:20)	Colorimetric	B	66
	Chloroform + Methanol + Ethyl Ether (75:15:10)	UV <sub>254</sub> or Colorimetric	B	67
	Cyclohexane + Benzene + Diethylamine (75:15:10)	UV <sub>254</sub> or Colorimetric	B	67
	Methanol + NH <sub>4</sub> OH (100:1.5)	UV <sub>254</sub> or Colorimetric	B	67
	n-Propanol + Chloroform + NH <sub>4</sub> OH (50:50:1.5)	UV <sub>254</sub>	T	68
	Benzene + Dioxane + Ethanol + NH <sub>4</sub> OH (50:40:5:5)	Colorimetric	T	69
	Ethanol (sat. w/NaCl) + Acetic Acid + Water (70:20:5)	Colorimetric	T	69
	Butanol + Acetic Acid + Water (60:15:25)	Colorimetric	U,B	70

TABLE XIV Continued

Silica Gel	Acetone + 1N NH <sub>4</sub> OH (1:1)	Colorimetric	U,B	70
	Chloroform + n-Propanol + NH <sub>4</sub> OH (100:100:2)	Colorimetric	T	71
	Chloroform + Toluene + Acetone (50:40:5)	Colorimetric	T	71
Alumina	Methanol + Chloroform (2.5:97.5)	Colorimetric	M	58
	Ethylene Dichloride + Ethanol (5:1)	Colorimetric	U,B	70

\*B = Blood, Plasma, Serum  
M = Microbiological Media  
T = Tissue  
U = Urine

TABLE XV

HPLC Methods

Column	Solvent System	Detection†	Source*	Reference
Partisil PXS-ODS	3.3 g $K_2HPO_4$ + 4.2 g $KH_2PO_4$ + 0.88 ml Acetic Acid + 1.2 l Water + 2.8 l Methanol	UV <sub>254,280</sub>	M	58
μ-Porasil	Methanol + 2N $NH_4OH$ + 1N $NH_4NO_3$ (27:2:1)	UV <sub>254,280</sub>	M	58
8 Micropak SI-5	Methylene Chloride + 2-Propanol + $NH_4OH$ (100:10:0.2)	UV <sub>240</sub>	U	72
Micropak SI-10	Chloroform + n-Propanol + $NH_4OH$ (100:100:2)	UV <sub>256</sub>	B,T	73
Hypersil-ODS	Acetonitrile + 10mM $Na_2HPO_4$ containing 80mM Sodium Lauryl Sulphate + 5mM Tetrabutylammonium Bromide (50:50)	UV <sub>254</sub>	B	74
Silica B-5	Methanol + Acetonitrile (1:5) + $NH_4OH$ 4 ml/l	Fl <sub>240/370</sub>	B	75
LiChrosorb SI-60	Ethyl Acetate + 0.05% Methylamine	UV <sub>254</sub>	B	76

TABLE XV Continued

Silica B-5	Acetonitrile + NH <sub>4</sub> OH (99.3:0.7)	UV <sub>211</sub>	B	77
LiChrosorb SI-60	Methylene Chloride + 0.2% Isopropanol + 0.45% n-propylamine	UV <sub>254</sub> FIMS	B	78
μ-Bondapak Phenyl	Acetonitrile + 0.015% Phosphoric Acid (71:29)	Fl <sub>252/360</sub>	B	79
Spherisorb-CN	Acetonitrile + pH 7 Phosphate Buffer + Methanol (45:35:20)	UV <sub>210,214</sub>	B	80
LiChrosorb RP-8, μ-Bondapak C <sub>18</sub> , Hypersil-ODS, LiChrosorb RP-18, Spherisorb-ODS	0.050M N,N-Dimethyloctylamine in Methanol + pH 3 Phosphate Buffer (1:1)	UV <sub>254</sub>	B	81

†Fl=Fluorescence; FIMS=Field Ionization Mass Spec.; UV=Ultraviolet Absorption

\*B=Blood, Plasma, Serum; M=Microbiological Media; T=Tissue; U=Urine

TABLE XVI

Gas Chromatographic Methods

Column	Column T (°C)	Detection <sup>†</sup>	Source*	Reference
2m x 2mm, 3% OV-17 on Gas Chrom Q	240°	MS	B	11
50cm x 6mm, 3% OV-17 on Gas Chrom Q	180°-220°	MS	B	12
1.5m x 2mm, 3% SP-2250DB on Supelcoport	205°	CIMS	B	13
2m x 2.5mm, SE-30 on Anakrom ABS	240°	FI	T	16
6ft x 2mm, 1% OV-17 on Supelcoport	220°	MS	B	42
6ft x 2mm, 5% OV-17 on Supelcoport	240°	N <sub>2</sub>	B	82
1.8m x 2mm, 1% OV-17 + 2% OV-225 on Chromosorb WHP	215°	N <sub>2</sub>	B	83
1.5m x 2mm, 3% OV-225 on Gas Chrom Q	220°	CIMS	B	84
1.8m x 2mm, 3% OV-17 on Gas Chrom Q	T <sub>i</sub> =240° for 2min. 32°/min to T <sub>f</sub> =265°	N <sub>2</sub>	B	85
2m x 2mm, 1.4% Carbowax 20M + 1.4% KOH on Gas Chrom Q	200°	FI	B	86
1.8m x 3mm, 2.5% OV-17	225°	FI or N <sub>2</sub>	B	87

TABLE XVI Continued

4ft x 2mm, 3% OV-17 on DMCS Treated Gas Chrom Q	T <sub>i</sub> =220° 8 <sup>1</sup> / <sub>6</sub> /min to T <sub>f</sub> =270°	MS	B	89
1.5m x 2mm, 1.5% Poly S-179 on Chromosorb W AW DMCS	230°-250°	MS	B	89
1.5m x 2mm, 3% OV-17 on Chromosorb W-HP	230°-250°	MS	B	89

†CIMS=Chemical Ionization Mass Spec.; FI=Flame Ionization; MS=Mass Spec.; N<sub>2</sub>=Nitrogen Detector

\*B=Blood, Plasma, Serum; T=Tissue

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# CISPLATIN

Christopher M. Riley

*University of Florida  
Gainesville, Florida*

Larry A. Sternson<sup>1</sup>

*University of Kansas  
Lawrence, Kansas*

1. History	78
2. Description	78
2.1 Nomenclature	78
2.2 Formulae	79
2.3 Molecular Weight	79
2.4 Appearance, Colors, and Odors	79
2.5 Elemental Composition	79
3. Synthesis	79
4. Physical Properties	81
4.1 Melting Point and Heat of Fusion	81
4.2 Dipole Moment	82
4.3 Ultraviolet and Visible Spectra	82
4.4 Mass Spectrum	82
4.5 Raman Spectra	85
4.6 Solubility	88
5. Analytical Methods	89
5.1 Electrochemistry	89
5.2 X-Ray Emission Spectrometry	91
5.3 Atomic Absorption Spectrometry	91
5.4 Ultraviolet and Visible Spectrometry	92
5.5 High Performance Liquid Chromatography	93
5.6 Paper Chromatography	96
6. Stability	96
6.1 Bulk Drug	96
6.2 Formulations and Aqueous Solutions	96
6.3 Biological Fluids	100
7. Pharmacokinetics	100
8. Biotransformation	101
References	101

<sup>1</sup>Present address: Smith Kline & French Laboratories, Philadelphia, Pennsylvania.

## 1. History

Dichlorodiammineplatinum (II) was first synthesized in 1845 by Peyrone (1) and was separated into its cis- and trans- isomers by Werner (2) in 1898. However, its cytotoxic properties were not reported until 1967 when Rosenberg et al (3,4) found that the application of an electrical field to a suspension of E. coli prevented cell division of the bacteria which continued to grow into long filaments. This inhibition of cell division was finally attributed to the presence of cis-dichlorodiammineplatinum (II) and cis-tetrachlorodiammineplatinum (IV) which were produced electrolytically from the platinum electrodes and the ammonium chloride used in the microbiological study (3,4). The initial animal studies revealed that both these platinum complexes had antineoplastic activity and that the platinum (II) complex, cisplatin, was more potent. Cisplatin entered clinical trials in the early 1970s and is now widely used for the treatment of various solid tumors of the head, neck, testes, ovaries, lung and bone (5). Up to the present time about 2,000 cisplatin analogues have been investigated as potential antineoplastic agents, in an attempt to improve the therapeutic ratio and to reduce the severe side effects of cisplatin which include nausea, vomiting, kidney damage and deafness.

## 2. Description

### 2.1 Nomenclature

#### 2.1.1 Chemical Names

cis-Diamminedichloroplatinum (II)  
cis-Dichlorodiammineplatinum (II)  
Platinum, diamminedichloro-, cis

#### 2.1.2 Generic Name

Cisplatin

#### 2.1.3 Trade Name

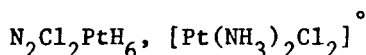
Platinol

### 2.14 Registry Numbers

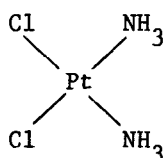
Chemical abstracts: 15663-27-1  
National Cancer Institute; NSC-119875

## 2.2 Formulae

### 2.21 Empirical and Chemical



### 2.22 Structural



## 2.3 Molecular Weight

300.09

## 2.4 Appearances, colors and odors

Cisplatin is a bright yellow crystalline powder with virtually no characteristic odors.

## 2.5 Elemental Composition

N(9.34%), H(2.02%), Cl(23.63%), Pt(65.01%)

## 3. Synthesis:

Cisplatin is generally synthesised (6-8) (Figure 1) by ammonolysis of potassium tetrachloroplatinate ( $\text{K}_2\text{PtCl}_4$ ) using methods based on that originally described by Peyrone (1). An aqueous solution of tetrachloroplatinate is prepared from the more readily available dipotassium hexachloroplatinate by reduction with potassium oxalate or hydrazine hydrochloride (6). Then, either, ammonia/ammonium chloride (6) or ammonium acetate, are used for the conversion of tetrachloroplatinate to cisplatin (7).

For example potassium tetrachloroplatinate (4.15 g) and conc. HCl (2.5 ml) are dissolved in water (75 ml). Ammonium chloride (3 g) is added to the solution,

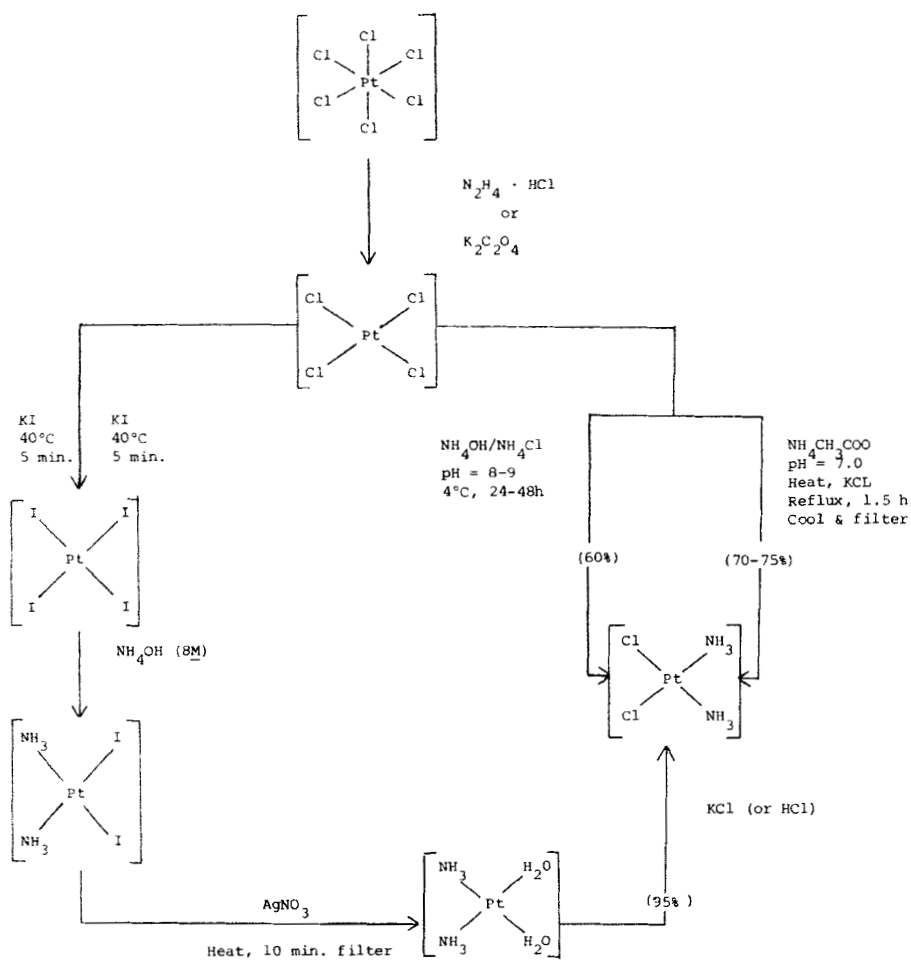


Figure 1 - Summary of synthetic procedures for cisplatin

followed by slow addition of 3 M ammonium hydroxide to bring the solution to pH 7.0. Further ammonium hydroxide (3 M, 6.75 ml) is then added and the solution refrigerated for 24 to 48 hours, during which time a greenish-yellow precipitate of cis-dichlorodiammine platinum (II) is formed. Purification is by recrystallization from 0.1 M HCl, ion exchange on a mixed bed resin to remove ionic impurities, or recrystallization from organic solvents such as DMSO/HCl, DMA/HCl or DMF/HCl. Golovnya and Lebedinskiĭ (7) have described a more rapid procedure for the ammonolysis of tetrachloroplatinate using ammonium acetate. Potassium tetrachloroplatinate (1 g) is dissolved in water and an aqueous solution (20%) of ammonium acetate (4 ml) is added. The resultant solution is heated and potassium chloride added just prior to boiling. The solution is refluxed for 1½ hours and then filtered. Orangish-yellow cis-dichlorodiammineplatinum (II) precipitates upon cooling. Purification methods are as described above. Dhara (8) has described an alternative method for the synthesis of cisplatin from potassium tetraiodoplatinate (figure 1). Potassium iodide (3.3 g) is added to an aqueous solution of potassium tetrachloroplatinate (2.0 g/20 ml) and heated for 5 minutes at 40°C. The solution turns dark blue due to the quantitative formation of potassium tetraiodoplatinate. Ammonium hydroxide (8 M, 1.5 ml) is then added causing formation and precipitation of cis-diiododiammine platinum (II) which is removed by filtration and washed with hot water, ice cold ethanol and ether. The cis-diiododiammine platinum (II) (2 g) is suspended in an aqueous solution of silver nitrate (1.4 g/10 ml) and heated on a steam bath for 10 minutes. The insoluble silver iodide is removed by filtration and the filtrate treated with a 10% excess of potassium chloride (or hydrochloric acid). Bright yellow crystals of cis-dichlorodiammineplatinum (II) appear after 10 to 15 minutes of heating on a water bath. Purification is as described above.

#### 4. Physical Properties

##### 4.1 Melting point and heat of fusion

M. pt. 270°C (decomposes)

$\Delta H^\circ_f$  477 kJ/mol (114 kcal/mol)



## 4.2 Dipole moment

5.3 D

## 4.3 Ultraviolet and visible spectra

The absorption spectrum (Figure 2) of cisplatin (1.0 mg/ml) was obtained in 0.1 N HCl using a Cary 15 and 2.0 cm cells. The spectrum shows three maxima at ca 203 nm, 301 nm and 362 nm, a "shoulder" at 285 nm and minima at 246 nm and 348 nm. There is negligible absorption above 480 nm. The extinction coefficients of cisplatin in 0.1 N HCl, at various wavelengths, are shown in Table 1.

Table 1

The Molar ( $\epsilon$ ) and Percentage ( $E_{1\text{cm}}^{1\%}$ ) Extinction Coefficients of Cisplatin

Wavelength (nm)	$\epsilon$ ( $\text{M}^{-1}\text{cm}^{-1}$ )	$E_{1\text{cm}}^{1\%}$ ( $\%^{-1}\text{cm}^{-1}$ )
203	5,200	173
285	190	3.63
301	130	4.33
362	24.2	0.806

## 4.4 Mass Spectrum

Cisplatin has generally been considered to be too involatile and unstable at high temperatures to be suitable for mass spectrometry. However, very recently, Weller et. al. (9) have obtained a positive ion mass spectrum of cisplatin using Fourier Transform mass spectrometry (FT-MS). The advantages of this technique lie in the very low pressures ( $10^{-8}$  torr) and the high sensitivity which can be achieved. Figure 3 shows the electron impact mass spectrum of cisplatin, obtained using a Nicolet Fourier Transform Mass Spectrometer (FT-MS 1000) with an Oxford Instruments 30 kG super conducting magnet. Solid samples were introduced through an airlock to a distance of 2 cm from a cubic sample cell and heated

Figure 2 - Ultraviolet and visible absorption spectrum of cisplatin (1 mg/ml)  
in 0.1 N HCl at 25°C

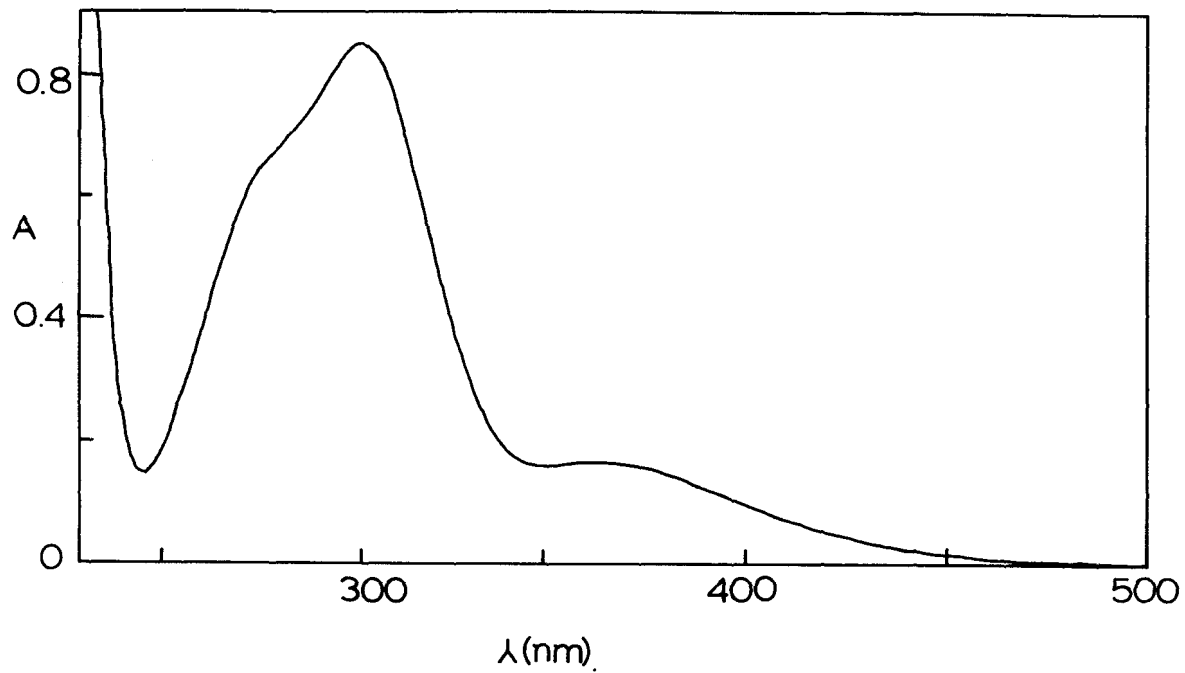
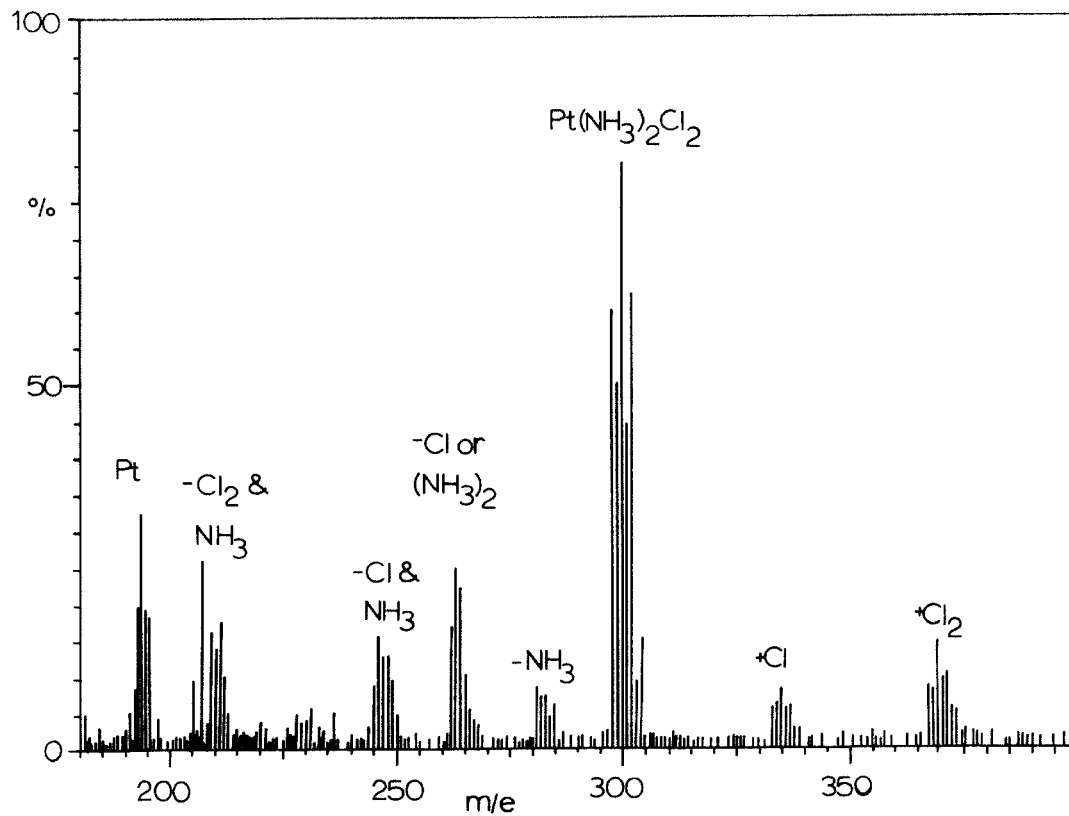


Figure 3 - Fourier Transform mass spectrum of cisplatin



in a stepwise manner (5°C steps). At about 300°C the spectrum was observed. One thousand transients each of 64K data points were obtained, averaged and transformed. A pressure of  $9 \times 10^{-8}$  torr was used.

The components of the isotopic clusters were within 1 to 2% of those predicted from the natural abundance of platinum and chlorine. Figure 4 shows the high resolution mass spectrum of cisplatin with the most abundant ion at  $m/e = 300$ . The fragmentation pattern (figure 3) was consistent with the sequential loss of the Cl and  $\text{NH}_3$  ligands. In addition to fragmentation, ion-molecule interactions between cisplatin and chloride were observed giving rise to  $[\text{Pt}(\text{NH}_3)_2 \text{Cl}_3]^+$  and  $[\text{Pt}(\text{NH}_3)_2 \text{Cl}_4]^{3+}$ . No ions were observed below  $m/e = 190$  except for those due to HCl.

Negative ion spectra were also obtained (9), however these generally gave less information and no molecular ions were observed.

#### 4.5 Raman Spectra

The detection of trace amounts of trans- $[\text{Pt}(\text{NH}_3)_2 \text{Cl}_2]$  in cisplatin via laser Raman spectroscopy is possible since the Pt-N and Pt-Cl symmetrical vibrational modes for the trans isomer (a control-symmetrical molecule having  $D_{2h}$  symmetry) are Raman active but infrared inactive whereas those for the cis-isomer ( $C_{2v}$  symmetry) are coincident for both the Raman and infrared. The Raman spectra of the pure cis and trans isomers are reproduced in Figure 5. The spectra were recorded using solid samples in capillary tubes, a 100 mW He-NE laser and a sophisticated spectrometer, consisting of a Spex 1400 Double Monochromator and ITT-FN-130 'star-tracker' photomultiplier, capable of  $1 \text{ cm}^{-1}$  resolution. The characteristic peaks of the pure compounds are readily detected in admixtures of the cis- and trans-isomers. However, when admixtures by intentional mixing are recrystallized from 0.1N HCl, two new bands appear at  $506$  and  $262 \text{ cm}^{-1}$ . These bands can be used to detect the presence of the trans-isomer in cisplatin samples and are clearly evident even at the 0.1% level for the  $262 \text{ cm}^{-1}$  frequency, but only at the 1% level when monitoring the  $506 \text{ cm}^{-1}$  band. These new bands could arise out of mixed crystal formation of trans in cis, a possible twinning arrangement, or other

Figure 4 - High resolution Fourier Transform mass spectrum of cisplatin

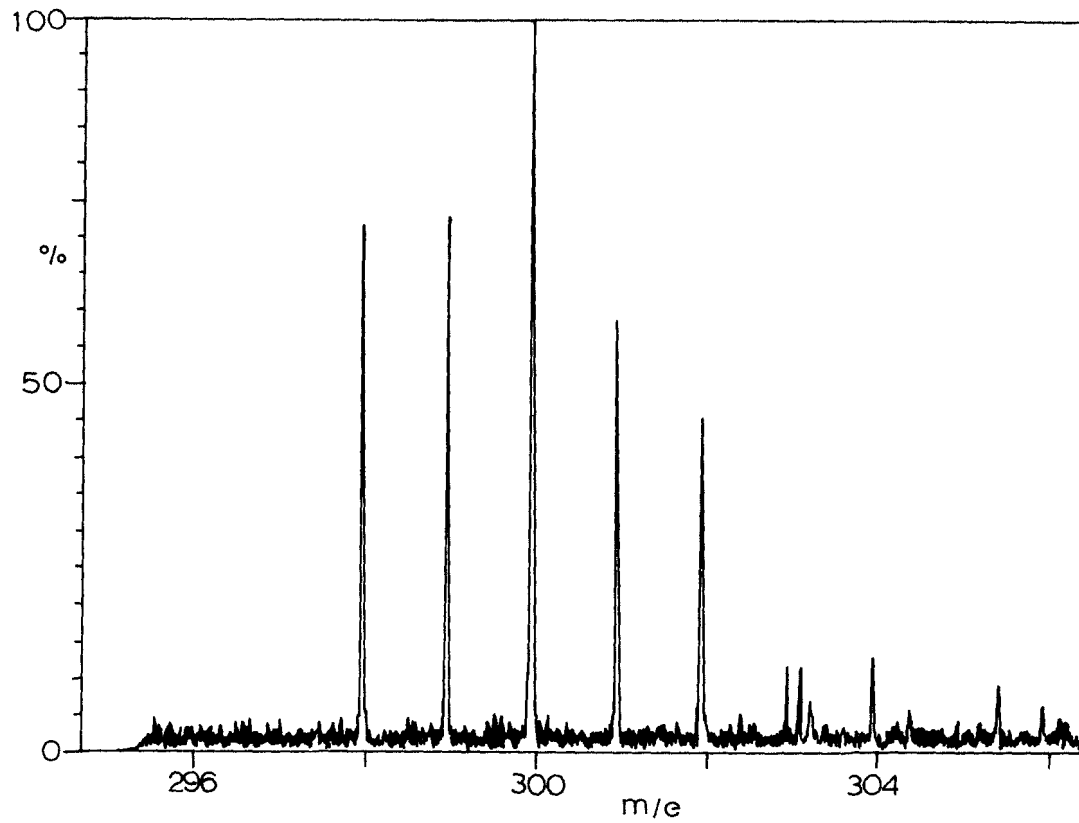
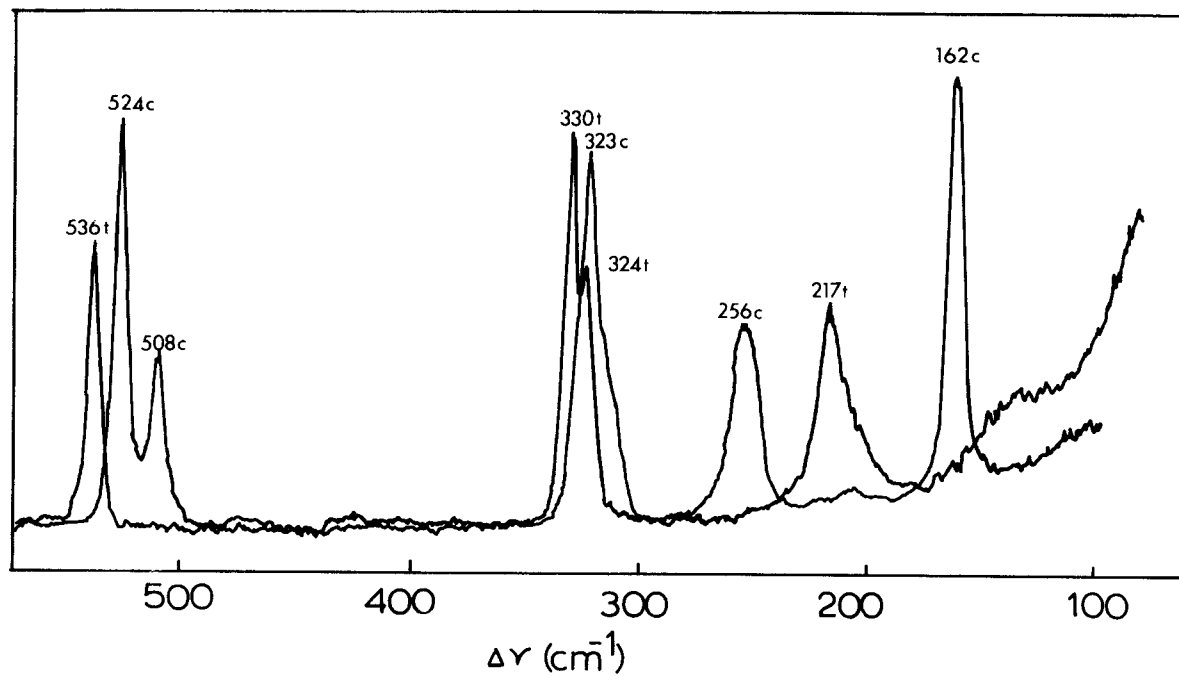


Figure 5 - Raman Spectrum of cis- and trans- dichlorodiammineplatinum (II).  
The letters (c and t) identify the peaks for the two isomeric forms.



crystallographic phenomena. The Raman frequencies of the pure compounds and a prepared mixture are compared in Table 2

Table 2

Raman Frequencies of cis- and trans-Pt(NH <sub>3</sub> ) <sub>2</sub> Cl <sub>2</sub> (pure components and mixtures)				
Complex	Pt-N	Pt-Cl		
<u>cis-</u>	524/508	323	257/255	162
<u>trans-</u>	536	330/324		217
<u>cis- + trans-</u>	506		262	

\* Only new frequencies are given, the other bands appear as expected for pure cis-isomer.

#### 4.6 Solubility

Cisplatin is insoluble in virtually all organic solvents except N,N-dimethylformamide (DMF), N,N-dimethylacetamide (DMA) and dimethylsulphoxide (DMSO). (Table 3). Cisplatin can undergo solvolysis as a result of nucleophilic substitution (10-18) and data based on non-specific determinations of platinum in solution (e.g. flameless atomic absorption spectroscopy) may only represent apparent solubilities. Due to aquation (sec. 6.2a), the apparent solubility of cisplatin in water is dependent on time. In the presence of excess chloride, greater than 99% of the platinum in solution will be present as intact cisplatin and the solubility is independent on time.

Table 3

## Apparent Solubilities of Cisplatin (25°C)

Solvent	Solubility		Ref
	M ( $\times 10^2$ )	% w/v (Cisplatin equivalents)	
DMF	6.67	2.0	19
DMA	6.00	1.8	19
DMSO	116.6	35.0	19
H <sub>2</sub> O (24 h)	0.77	0.23	20
(72 h)	0.83	0.25	20
0.9% NaCl	0.50	0.15	20

5. Analytical Methods5.1 Electrochemistry

Electrochemical measurements have been infrequently applied to the determination of platinum complexes. Although platinum (II) can be oxidized and reduced at electrodes composed of various materials (21) these reactions are very dependent on the identity of the ligands coordinated with platinum (21) and are subject to electrode surface phenomena (22) and reaction medium effects (22). Coulometric titrimetry (23) inverse stripping voltammetry (24) and platinum catalysis of the hydrogen discharge at a mercury electrode (25) are the few example of techniques used to determine platinum in solution.

Platinum (II) complexes are poorly behaved depolarizers, interacting strongly with the electrode surface (26,27). The reduction of these compounds



involves a two electron transfer to the central atom of an adsorbed molecule (27,28). Polarograms of chloro- and chloroammino platinum (II) complexes include maxima and minima (28,29) resulting from these surface effects. Platinum metal, adsorbed onto the electrode surface as growing crystal nuclei, is produced in the irreversible reduction (30). The electro-deposited metal alters the advantageous hydrogen over-potential of mercury causing catalytic hydrogen currents at potentials as anodic as -600 mV (SCE) (31). Electro-oxidations of platinum (II) complexes occur through bridging halide and are quite slow in the absence of free halide (32).

Because of these analytically unsuitable electrode reactions, voltammetric methods have not been frequently used for the determination of platinum. In one reported electrochemical method, the catalytic hydrogen current resulting from platinum metal deposition is used quantitatively in a sensitive differential pulse platinum determination at mercury (33).

Polarography has recently been used as a means of HPLC detection for post-column monitoring of cisplatin in biological fluids (34). Many of the problems which make electrochemistry unsuitable for platinum analysis in solution can be ameliorated by the controlled environment of the HPLC effluent and the separation of platinum species from each other and from sample matrix components. Using solvent-generated anion exchange HPLC with totally aqueous mobile phase (35), both dropping mercury (DME) and hanging mercury drop (HMDE) cathodes have been successfully adapted for cisplatin analysis (34). Column effluent is passed into an electrochemical detector consisting of a mercury drop polarized at 0.00 V (vs Ag/AgCl) in a cell thermostated at 60°C. Oxygen must be eliminated from the system by appropriate argon purging (which has been scrubbed with vanadous chloride (34,36)). The noise-limited minimum detectable quantities of cisplatin with DME and HMDE are 1.8 ng and 70 pg injected, respectively. Cisplatin can be determined in untreated biological fluid at levels of 70ng/ml (34).

## 5.2 X-Ray Emission Spectrometry

Proton-induced X-ray emission spectrometry has been used (37,38) for platinum detection in biological samples, but has not been applied to clinical investigations due to the laborious sample preparation required. Platinum derived from cisplatin has been determined in clinical samples by wavelengths dispersive X-ray fluorescence (39). Following its separation from protein bound platinum by centrifugal ultrafiltration, the lower molecular weight platinum-containing species ( $MW < 25,000$ ), are derivatized with ethylenediamine and the resulting cationic product (i.e., representing the reactive platinum in the ultrafiltrate) retained on a cellulose disc impregnated with cation exchange resin. The platinum is counted directly on the disc supported between two sheets of polypropylene film. Instrument parameters: X-ray tube molybdenum, operated at 50 kV & 50 mA; analyzing crystal,  $LiF_{220}$ ; goniometer,  $PtL_{2\theta}$ , 54.91 2 $\theta$ , background 23.70 2 $\theta$ . This method offers a detection limit of 240 ng of cisplatin/ml of plasma at the 3s level with fluorescence intensity linearly related to drug concentration over the range 570 to 5700 ng/ml.

## 5.3 Atomic Absorption Spectrometry

Non-flame atomic absorption (NFAA) spectrometry is the most commonly used technique for total platinum determinations, particularly in biological samples (40-52). Using electrothermally heated graphite tubes as the atomic vapor source, NFAA offers a significant improvement in sensitivity over flame methods (53). Instrumental analysis of prepared samples is fast and convenient. In the absence of interferences, NFAA can determine platinum at 1 ng/ml in aqueous samples (54). Platinum, however, is a relatively nonvolatile element and furnace temperatures of 2300 to 2700°C (55,56) (dependent on instrument design) are required for its atomization. Such temperatures cause decomposition of the pyrolytic graphite surface of the atomizer and subsequent imprecision and inaccuracy in the determination (57). Replacement of the atomizer (after 20-100 measurement cycles (54)) is required for signal quality restoration. Components of biological sample more commonly cause physical

changes in platinum atomization than spectral interferences (49). These matrix effects cause sample deposition changes (minimized by the addition of surfactant to the sample (49)) and interference due to smoke production upon atomization. These effects may be corrected by control of the temperature ramp rates (49)) and the use of a continuum-source second beam for background blanking (58). Biological fluids and tissues have been prepared for NFAA platinum analysis by homogenization (49), acid digestion (45) and furnace ashing (50).

This technique has also been utilized for detection of platinum-species derived from cisplatin following separation by HPLC (sec. 5.5). A procedure more sensitive than the previously reported X-ray fluorescence (39) method has been described for determination of free-circulating platinum species (i.e., non-protein bound) in plasma (59). Following the same sample preparation scheme described in X-ray emission, the platinum is eluted from the cation-exchange resin impregnated disc with 5 M HCl and an aliquot of this acid solution is introduced into the carbon rod furnace. The 265.95 nm platinum line is monitored.

Off-line NFAA has also been used for the detection of platinum eluted from HPLC columns (sec. 5.5) for the analysis of cisplatin in biological materials (35) and its reaction products (61). This technique is capable of locating and quantifying the platinum containing bands eluting from the column (61). Following chromatography (sec. 5.5), fractions are collected and monitored for total platinum by NFAA. Under these conditions, matrix effects are negligible.

#### 5.4 Ultraviolet and Visible Spectrometry

The purity of cisplatin samples may be evaluated using UV/visible spectroscopy by determining a) the molar absorbance at 301 nm, b) the position of the spectral minimum  $A(\min)$ , and c) the absorbance ratio,  $A(301 \text{ nm})/A(\min)$  (16,20,62). Cisplatin obeys Beer-Lambert's law up to its limiting solubility (1.5 mg/ml) in 0.1 N HCl, at 301 nm and its molar extinction coefficient is  $130 \pm 2$  (20). However, the determination of purity based on spectroscopic measurements made at a single wavelength (301 nm) is relatively insensitive to the presence of impurities

(16,20). The position of the minimum (246 nm for pure samples) and the absorbance ratio,  $A(301\text{ nm})/A(\text{min})$ , are much more sensitive to the presence of impurities since most Pt (II) and Pt(IV) complexes absorb around 246 nm. The presence of impurities generally results in a red shift of the minimum and a decrease in the absorbance ratio. Lee and Martin (16) have suggested that a value of greater than 4.5 for this absorbance ratio is a good criterion for purity.

The degradation (aquation, sec. 6.2a) of cisplatin may be monitored by UV spectroscopy which reveals a decrease in the absorbance at 301 nm with time. A sharp isosbestic point is not observed presumably due to the presence of multiple reaction products. Hussain et al (63) have used ultraviolet spectroscopy to monitor the reaction of cisplatin with bisulfite. The reaction of cisplatin with bisulfite produces intensely UV absorbing species utilized by Marsh et al (64) in a post column reaction detector for the analysis of cisplatin by HPLC (sec 5.5).

## 5.5 High Performance Liquid Chromatography

A number of HPLC procedures have been described for the analysis of cisplatin in drug formulations (13,65,66), reaction mixtures (61,67,68), urine (34,35,69-72) and plasma (50,72-74). The HPLC methods for cisplatin may be described as being specific, responding to the intact drug, or non-specific, responding only to total levels of platinum.

### a) Non-specific Methods

Bannister et al (69) and Borch et al (70) have described virtually identical methods for determining the platinum excreted in urine, following IV administration of cisplatin. These techniques rely on the stoichiometric reaction of Pt(II) with diethyldithiocarbamate to yield a neutral complex which may be extracted into chloroform.

The complex formed has a molar extinction coefficient of 43,000 at 254 nm and may be determined either directly by UV spectrophotometry or by HPLC. For the HPLC, Bannister et al (69) employed a  $\mu$ Bondapak CN column with a mobile phase of n-heptane:

2-propanol (82:18), whereas Borch et al (70) preferred a reversed phase system with a Varian MCH column (C18) and a mobile phase of acetonitrile:water (60:40). Both methods (69,70) gave identical detection limits which were equivalent to 25 ng/ml of cisplatin in urine.

b. Specific Methods

Cisplatin cannot be analyzed by adsorption chromatography (straight phase) due to its low solubility in the organic solvents used in these systems. Conversely, cisplatin is poorly retained in reversed phase systems (75), even when purely aqueous mobile phases are used.

The first method for the determination of intact cisplatin by HPLC can be attributed to Chang et al (61) who employed a strong cation exchange column (Partisil 10SAX) and a mobile phase of methanol:acetate buffer (0.1 M pH 3.8) (1:1). This method was applied to the analysis of plasma (61, 73 and 74) and formulations (13). By coupling the strong anion exchange with a cation exchange (Partisil 10SCX) the positively charged aquation products (sec. 6.2) may be determined in the presence of cisplatin (13).

The use of chemically bonded anion exchange has been associated (71) with poor peak shape for cisplatin and column instability. Additionally, the high concentration methanol (>50%) required for adequate retention of cisplatin results in precipitation of urine components upon injection (35) and are incompatible with polarographic (34) and reaction (64) detectors. A more flexible and reliable approach to the HPLC of cisplatin is that taken by Riley et al (35,68,71,72,75,76) who have employed solvent generated anion exchanges, prepared by adsorbing a cationic surfactant, hexadecyltrimethylammonium bromide, onto the surface of a reversed phase column. These systems offer improved peak shape, better reproducibility and column stability compared with chemically bonded anion exchangers. Furthermore, cisplatin is retained on solvent generated anion exchanges in the presence of purely aqueous mobile phases which are compatible with the direct injection of urine and with polarographic (34) and reaction (64) detectors.

The retention of cisplatin on chemically bonded anion exchangers (e.g. Partisil 10SAX) is controlled by varying the concentration of organic modifier (usually methanol) in the mobile phase (75). In contrast the retention of cisplatin on solvent generated anion exchangers is controlled by the concentration and nature of the electrolytes added to the aqueous mobile phase (76). The retention of cisplatin and other neutral platinum complexes (35) on positively charged stationary phases is believed to arise as a result of ion-dipole interactions.

Mariani et al (65) have used a  $\mu$  Bondapak  $\text{NH}_2$  column with a mobile phase of acetonitrile:water (9:1) for the analysis of cisplatin in drug formulations and Klein et al (62) have described a similar method for the separation of the cis and trans isomer of cisplatin.

Riley et al (61,64) have described HPLC systems for following the reaction of cisplatin with peptides (69), methionine (68) and nucleotides (68). Solvent generated anion exchangers were used to study the reaction of cisplatin with nucleotides (68). However, gradient elution (acetonitrile 0-60%) was required to elute the product formed from the reaction of cisplatin with peptides (68). The reaction of cisplatin with methionine produces cationic compounds which can be retained on a reversed phase column by the addition of alkylsulphates as pairing ions to the mobile phase (68).

The detection limit for cisplatin in HPLC systems using UV detectors is about 10 to 50  $\mu\text{g/ml}$ , depending on conditions. Cisplatin may be determined by HPLC at levels of 50  $\text{ng/ml}$  if the appropriate eluant fraction is collected (35,60) and analysed by NFAA (sec. 5.3). Off-line detection of cisplatin is time consuming and recently two on-line detection systems have been described (35,64). Bannister et al (34) have described a polarographic HPCL detector which permits detection limits of 70 $\text{ng/ml}$  of cisplatin in urine. Marsh et al (64) have described a post column reaction detector in which cisplatin is reacted with bisulphite, in the presence of

dichromate, after elution from the column. The formed complex absorbs strongly at 290 nm, permitting detection limits of 40 ng/ml in biological fluids. Both the polarographic (34) and bisulphite reaction detectors (64) employ solvent generated anion exchanger for the chromatographic steps.

## 5.6 Paper Chromatography

Basolo et al (77) have described a paper chromatography method for the separation of cisplatin from its trans isomer. The separation is performed on cellulose filter paper which was developed for one to two hours with either butanol:water or ethanol:water mixtures. Substitution reactions (presumably aquation) were minimized by carrying out the separation at 4°C and the inclusion of 1 N HCl in the eluant. The authors (77) report very variable  $R_f$  values of the complexes and recommend that reference substances be run on the same paper as the samples to be tested. The spots were identified after separation by development with iodine vapors.

## 6. Stability

### 6.1 Bulk Drug

cis-Dichlorodiammineplatinum (II) has been found stable to light and air at room temperature, but degrades to platinum metal at 270°C (20).

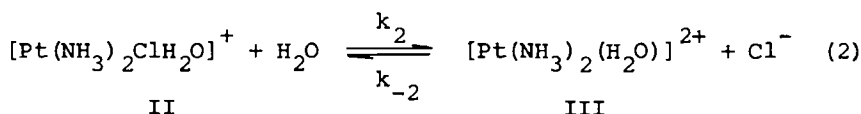
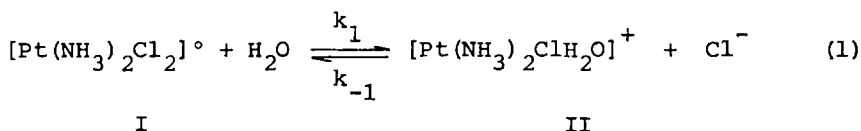
### 6.2 Formulations and Aqueous Solutions

Cisplatin is supplied as a freeze dried powder containing mannitol and sodium chloride. The intact vials have shelf lives of 2 and 4 years at room (22-25°C) and refrigeration (2-8°C) temperatures, respectively. When reconstituted with Sterile Water for Injection USP, each 1 ml should contain 1 mg cisplatin, 9 mg NaCl and 10 mg mannitol and the resulting pH should lie between 3.5 and 5.5 (78).

Potential instability of the reconstituted injection and aqueous solution may arise from a) aquation, b) incompatibilities of formulatary adjuvants and co-administered drugs, c) contact with metal surfaces and d) microbial contamination.

a) Aquation

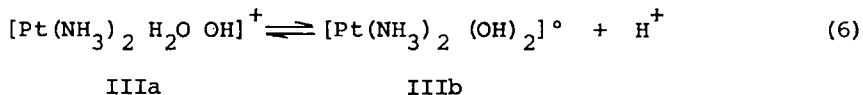
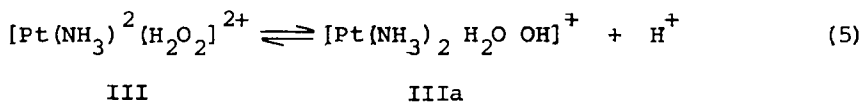
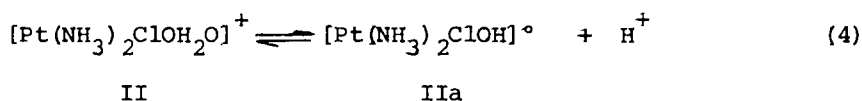
Cisplatin (I) undergoes aquation in aqueous solution (10-18) to produce the aquation products shown in equations 1 and 2



Assuming that the loss of cisplatin is not influenced significantly by equation 3, then the appropriate rate equation is:

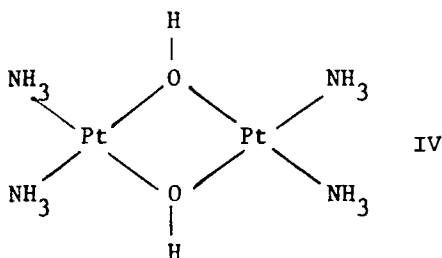
$$-\frac{d[\text{I}]}{dt} = k_1 [\text{I}] - k_{-1} [\text{II}] [\text{Cl}] \quad (3)$$

A value of  $0.089 \text{ h}^{-1}$  has been reported for  $k_1$  at  $25^\circ\text{C}$ , by various authors (13,15,19). The aquation products (II and III) are weak acids capable of dissociation in aqueous solution (equations 4-6).





Unlike the positively charged aquation products (II, III, IIIa) which are highly reactive toward "soft" nucleophiles (10), the neutral hydroxylated complexes (IIa, IIIb) are "stable". In addition to the aquation products described above, "bridged" hydroxylated complexes (IV) have been reported (79,80). Under acidic conditions,



and in the absence of added chloride, the rate of loss of cisplatin and the concentration of drug at equilibrium is dependent on its initial concentration (13). As the pH increases the fraction of the monoaquated complex (II) ( $pK_a = 6.0$ ) present as the neutral hydroxylated species (IIa) increases. Since the hydroxylated complex (IIa) is highly stable to nucleophilic substitution, the influence of initial concentration of cisplatin on the rate of degradation and concentration of cisplatin at equilibrium decreases with increasing pH. This was confirmed by Hincal *et al* (13) who showed that the addition of 5% w/v sodium bicarbonate (pH 7.5) increases the rate of degradation of cisplatin and lowers the concentration present at equilibrium.

The addition of chloride to aqueous solution of cisplatin influences the rate of degradation (equation 3) and more importantly the concentration of drug at equilibrium. Table 4 shows the percentage of drug present after 12 hours in solutions containing various concentrations of sodium chloride (13). These data indicate that almost 1% of the drug would be lost from the reconstituted injection over this 12 hour period. Note. The shelf life of the

reconstituted products is 8 hours at room temperature (sec 6.2d).

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Table 4

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Percentage of Drug Present in Solutions Originally Containing Cisplatin (50 and 500 µg/ml) and Various Concentrations of Sodium Chloride after 12 hours at 25°C (pH 7.5).

Sodium Chloride Concentration (% w/v)	Cisplatin Remaining (%)	
	[I] <sub>0</sub> = 50 µg/ml	[I] <sub>0</sub> = 500 µg/ml
0.00	36.5	39.0
0.10	86.0	89.0
0.45	98.0	98.0
0.90	99.0	99.0

---

From data by Hincal et al (13).

b. Incompatibilities

In addition to aqutation, cisplatin can undergo nucleophilic substitution by strong nucleophiles (10,67) in aqueous solution, indicating that the drug should not be formulated in the presence of such species. For example, bisulphite has been shown to react rapidly with cisplatin, suggesting that its inclusion as an antioxidant in formulations containing cisplatin is not recommended. Cisplatin is stable in the presence of mannitol (13), dextrose (13,65), benzyl alcohol (65) and parabens (65). As well as influencing the rate of aqutation the addition of sodium bicarbonate has been associated with the production of a bright gold colored precipitate on standing (78).

c. Metal Surfaces

Cisplatin is incompatible with aluminium producing a black precipitate on standing (78). There are no indications of its incompatibility with stainless steel.

#### d. Microbial Contamination

Commercial formulation of cisplatin contain no preservatives and the reconstituted injections have a shelf life of eight hours at room temperature (78).

#### 6.3 Biological Fluids

Due to the presence of endogenous nucleophiles, cisplatin is unstable in plasma (73,74), plasma ultrafiltrate (73,74) and urine (35). Consequently, biological fluids containing cisplatin should be frozen immediately after sampling, over dry ice/acetone, and stored at  $-11^{\circ}\text{C}$  in the cases of plasma (73) and plasma ultrafiltrate (73) and at  $-60^{\circ}\text{C}$  in the case of urine (35), if determinations of the intact drugs are to be performed. Subsequent HPLC mediated isolation of cisplatin should be carried out within 72 h, even when samples are stored at recommended temperatures. Storage at reduced temperatures is less critical when non-specific determination of total platinum concentrations only are to be performed on the samples.

#### 7. Pharmacokinetics

Gill *et al* (81) have monitored the total platinum concentrations by flameless atomic absorption spectrometry, in the plasma of nine patients who had received cisplatin ( $100 \text{ mg/m}^2$ ) for the treatment of various neoplastic diseases. They found biphasic elimination of the platinum from plasma (half lives of 34-46 h and 132-340 h), following a rapid, short lived, initial decline. A similar study was performed by Himmelstein *et al* (82) who, in addition to monitoring the total platinum concentration in plasma, also separated the plasma ultrafiltrate (m.wt. 25,000 and determined the concentrations of intact drug and total filterable platinum. The intact cisplatin was determined in the ultrafiltrate by HPLC with detection off-line using flameless atomic absorption spectrometry (67). The total filterable platinum was determined by direct analysis of the ultrafiltrate using flameless atomic absorption

spectrometry. Unlike the levels of total platinum which decline very slowly after IV administration of cisplatin, filterable species are eliminated rapidly from the kidney into the urine in a monoexponential manner ( $t_{1/2} = 30$  mins) and the disappearance of these species from plasma is paralleled by their appearance at similar concentration ratios in the urine (35,71). These observations are indicative of extensive and essentially irreversible protein and tissue binding of the platinum derived from an intravenous infusion of cisplatin. This has been supported by equilibrium dialysis experiments (74) and by the observation that only 10-30% of the dose appears in the urine in the first 24 hours (35,71).

#### 8. Biotransformation

At present none of the biotransformation products of cisplatin have been identified (83). However, the susceptibility of cisplatin to nucleophilic substitution strongly suggest that reaction with endogenous nucleophilic maybe responsible for the biotransformation of the drug (5,61,68) This hypothesis has been supported by in vitro studies which have shown that cisplatin reacts particularly rapidly with endogenous nucleophiles methionine, methionine containing peptides and other substances containing divalent sulfur (5,61,68).

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# TRIPLENNAMINE HYDROCHLORIDE

Hazel G. Piskorik

*CIBA-GEIGY Corporation  
Suffern, New York*

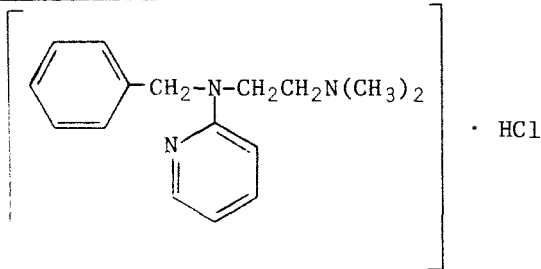
1. Description	108
1.1 Introduction	108
1.2 Formula, Name, Formula Weight	108
1.3 Appearance	108
2. Physical Properties	109
2.1 Ultraviolet Absorption Spectrum	109
2.2 Infrared Absorption Spectrum	109
2.3 Proton Nuclear Magnetic Resonance Spectrum	112
2.4 Carbon-13 Nuclear Magnetic Resonance Spectrum	114
2.5 Mass Spectrum	117
2.6 Fluorescence	118
2.7 Melting Range	119
2.8 Differential Scanning Calorimetry	119
2.9 Thermogravimetric Analysis	119
2.10 X-Ray Powder Diffraction	119
2.11 Microscopy	119
2.12 Dissociation Constant	122
2.13 Solubility	122
2.14 Partition Coefficients	122
3. Synthesis	123
4. Stability	124
5. Pharmacokinetics and Metabolism	124
6. Analytical Methods	125
6.1 Elemental Analysis	125
6.2 Nonaqueous Titration	126
6.3 Phase Solubility Analysis	126
6.4 Thin-Layer Chromatography	126
6.5 Gas Chromatography	126
6.6 Gas Chromatography-Mass Spectrometry	128
6.7 High Pressure Liquid Chromatography	129
6.8 Column Chromatography	129
6.9 Ultraviolet Spectrometry	129
6.10 Colorimetric	129
References	131

## 1. Description:

### 1.1 Introduction:

Tripeleennamine hydrochloride is an antihistamine, which acts as a competitive antagonist of histamine at  $H_1$  receptors (1-4).

### 1.2 Formula, Name, Formula Weight:



Formula Weight: 291.82  
 Formula:  $C_{16}H_{21}N_3 \cdot HCl$   
 Chemical Abstracts Number: 154-69-8

The following chemical names have been used to describe tripeleennamine hydrochloride (1,2,3,5):

- i. 1,2 Ethanediamine, N,N-dimethyl-N'-(phenylmethyl)-N'-2-pyridinyl-, monohydrochloride
- ii. 2-[Benzyl[2-(dimethylamino)ethyl]amino]pyridine hydrochloride
- iii. N-Benzyl-N'N'-dimethyl-N-pyrid-2-ylethylenediamine hydrochloride

Trade Names (6): Pyribenzamine Hydrochloride, PBZ-SR, Azaron, Benzoxal, Piristina, Pyrizil

### 1.3 Appearance:

Tripeleennamine hydrochloride is a white odorless crystalline powder with a bitter taste (3).

## 2. Physical Properties:

### 2.1 Ultraviolet Absorption Spectrum:

The ultraviolet absorption wavelength maxima ( $\lambda_{\max}$ ),  $A(1\%, 1\text{cm})$ 's) and molar absorptivities of tripeleennamine hydrochloride in three solvents are given in Table I. A typical spectrum of the compound in methanol obtained on a Perkin-Elmer Model Lambda 5 spectrophotometer is given in Figure 1.

Table I

Solvent	$\lambda_{\max}$ (nm)	$A(1\%, 1\text{cm})$	$\epsilon \times 10^{-3}$
0.1N HCl	313	278	8.11
	238	478	13.95
Water	305	164	4.78
	244	496	14.47
Methanol	307	157	4.58
	246	566	16.25

### 2.2 Infrared Absorption Spectrum:

The infrared absorption spectrum of tripeleennamine hydrochloride in a Nujol mull was obtained on a Perkin-Elmer Model 281B grating infrared spectrophotometer and is presented in Figure 2. Assignments for the characteristic bands in the spectrum are listed in Table II and are consistent with the structure.

Table II

Wavenumber ( $\text{cm}^{-1}$ )	Assignments
2600 - 2400	Tertiary amine salt
1596 1493 } 1435	Aromatic ring vibration; C=C; C=N
1368	Aryl C-N stretch

Figure 1  
Ultraviolet Absorption Spectrum of  
Tripelennamine Hydrochloride in Methanol

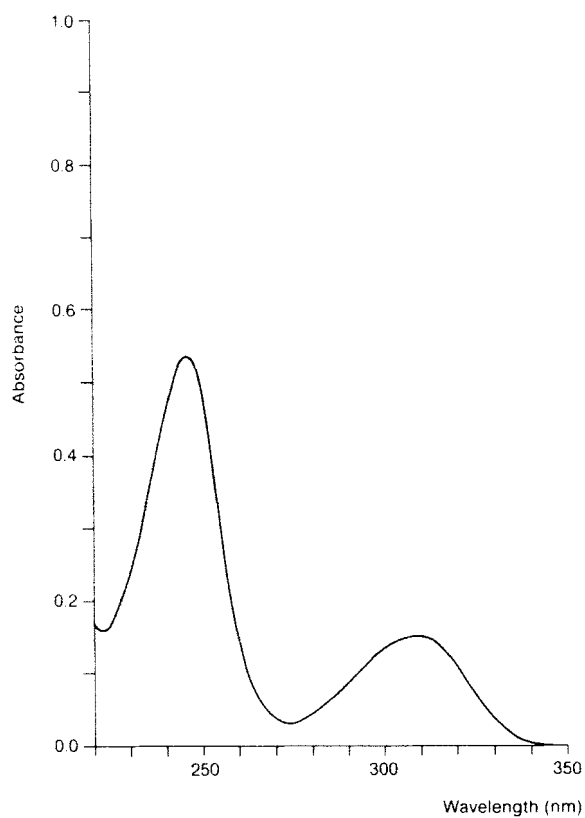


Figure 2  
Infrared Absorption Spectrum of Tripeleannamine Hydrochloride in Nujol

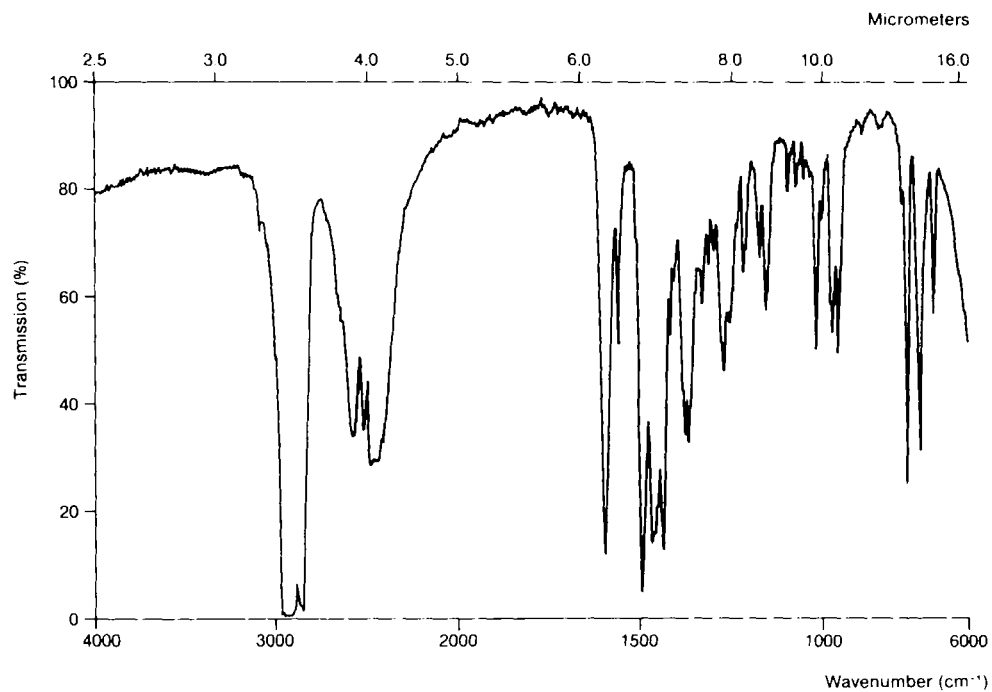


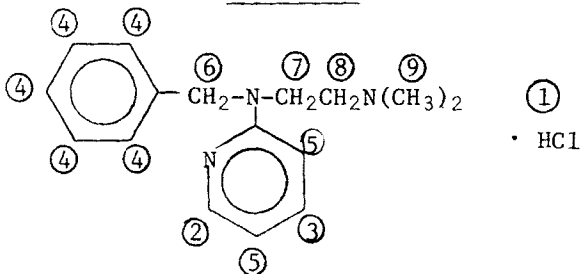
Table II  
(continued)

Wavenumber (cm <sup>-1</sup> )	Assignments
769	Monosubstituted benzene and 2-monosubstituted pyridine
731	2-Monosubstituted pyridine
695	Monosubstituted benzene

### 2.3 Proton Nuclear Magnetic Resonance Spectrum:

The 90 MHz proton nuclear magnetic resonance spectrum of tripeleennamine hydrochloride obtained in deuterated chloroform using tetramethylsilane as an internal standard is given in Figure 3. The spectrum was obtained on a JEOL FX90Q instrument at ambient temperature. The chemical shifts, multiplicities and spectral assignments are provided in Table III (7).

Table III



Proton Position	Chemical Shift (ppm)	Number of Protons	Multiplicity
1	11.55-12.05	1	Broad Singlet
2	8.14	1	Doublet
3	7.41	1	Multiplet
4	7.23	5	Multiplet
5	6.55	2	Multiplet

Figure 3  
90 MHz  $^1\text{H}$  Nuclear Magnetic Resonance Spectrum of Tripelennamine Hydrochloride  
at Ambient Temperature

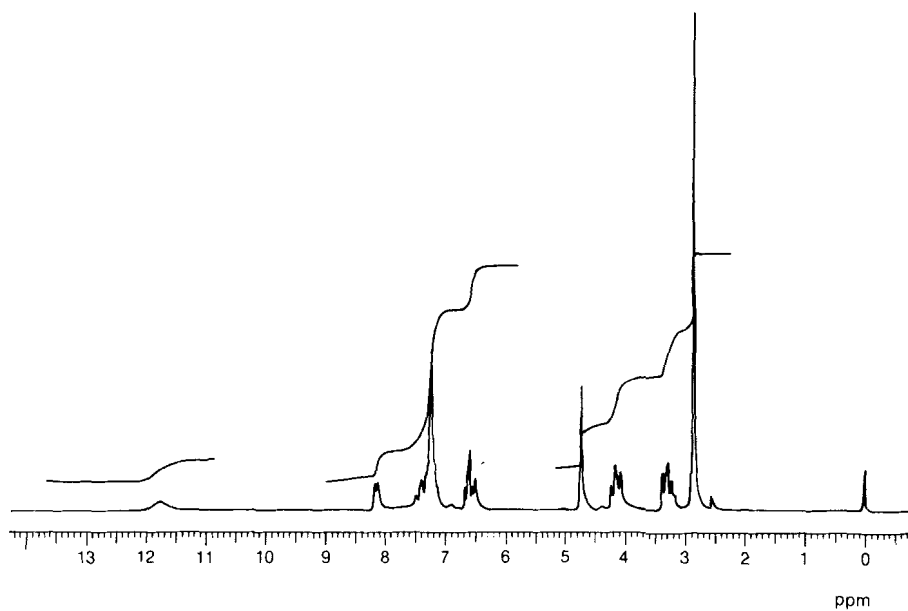


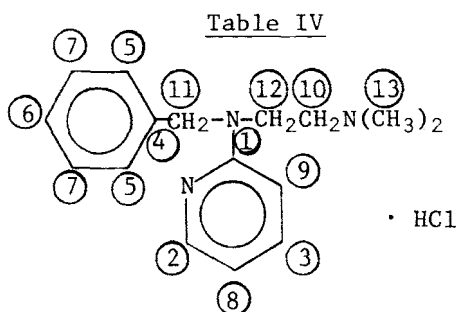


Table III  
(continued)

Proton Position	Chemical Shift (ppm)	Number of Protons	Multiplicity
6	4.73	2	Singlet
7	4.16	2	Triplet
8	3.29	2	Triplet
9	2.86	6	Singlet

#### 2.4 Carbon-13 Nuclear Magnetic Resonance Spectrum:

The  $^{13}\text{C}$  nuclear magnetic resonance spectrum of tripelennamine hydrochloride was obtained in deuterated chloroform at ambient temperature with  $^1\text{H}$ -decoupling. The spectrum given in Figure 4 was recorded at 22.5 MHz using TMS as the internal standard on a JEOL FX90Q instrument. The chemical shifts, multiplicities and spectral assignments are given in Table IV (7).



Carbon Position	Chemical Shift (ppm)
1	157.5
2	147.6
3	137.7
4	137.4
5	128.7
6	127.2

Figure 4  
22.5 MHz  $^{13}\text{C}$  Nuclear Magnetic Resonance Spectrum of Tripelennamine Hydrochloride  
with  $^1\text{H}$ -decoupling

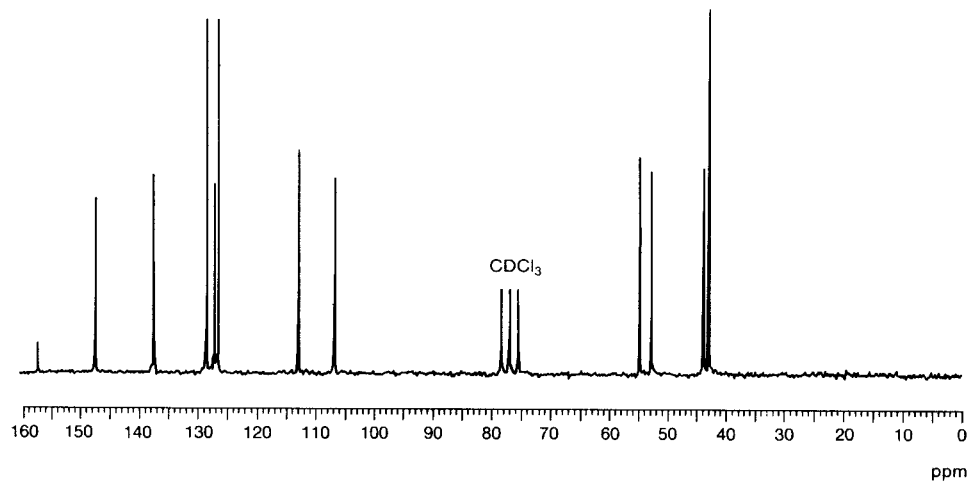


Figure 5  
Low Resolution Mass Spectrum of Tripeleppamine Hydrochloride

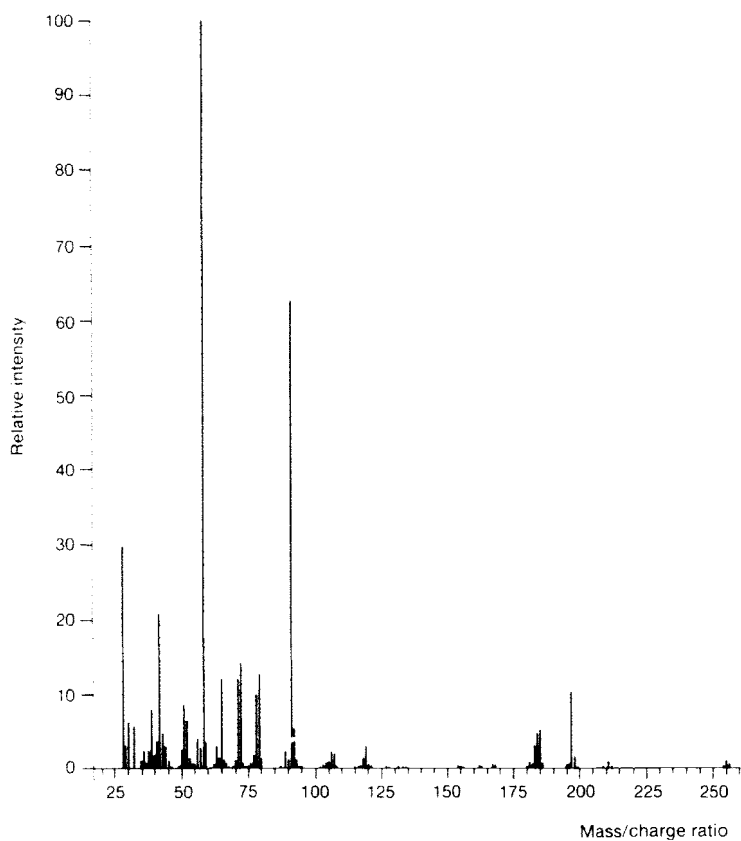


Table IV  
(continued)

Carbon Position	Chemical Shift (ppm)
7	126.8
8	113.1
9	106.9
10	55.0
11	53.0
12	44.0
13	43.1

### 2.5 Mass Spectrum:

The low resolution electron impact mass spectrum of tripeleennamine hydrochloride (Figure 5) was obtained on a Kratos MS 25 spectrometer. The electron energy was 70 eV and the sample was introduced into the ion source by a solid insertion probe at 100°C. The proposed fragmentation pattern and the corresponding mass/charge ratio is given in Table V (8). The observed spectrum which was recorded in the positive ion mode is that of the free base of the compound resulting from thermal dissociation when the compound is vaporized.

Table V

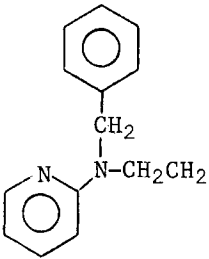
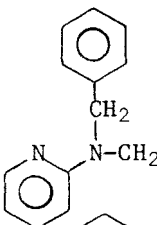
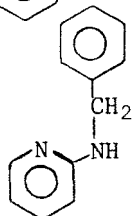
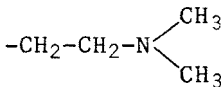
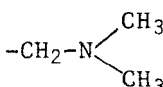
m/z	Fragment
256	M + 1
255	M (molecular ion)
211	

Table V  
(continued)

m/z	Fragment
197	
184	
91	Tropylium ion
72	
58	

## 2.6 Fluorescence:

Tripeleennamine hydrochloride exhibits both a natural fluorescence (9) and yields fluorescent products when treated with both cyanogen bromide (10,11) and hydrogen peroxide (11). Table VI summarizes the excitation and emission wavelengths for these various compounds.

Table VI

	Excitation	Emission
Natural Fluorescence	248,306	363
After CNBr	355	419
After H <sub>2</sub> O <sub>2</sub>	345	408

Onishi and co-workers determined the structure of the fluorescent product produced when tripelennamine hydrochloride is treated with cyangogen bromide to be 1-benzyl-2,3-dihydro-imidazo[1,2-a]pyridinium bromide (12).

#### 2.7 Melting Range:

The USP melting range specification for tripelennamine hydrochloride is between 188 and 192°C when tested according to the USP XX Class Ia procedure (13). A sample of tripelennamine hydrochloride USP reference standard was observed to melt between 189.0 and 189.5°C (14). The Merck Index (2) reports a melting range of 192.5 to 193.5°C for the compound.

#### 2.8 Differential Scanning Calorimetry:

The differential scanning calorimetry thermogram of tripelennamine hydrochloride obtained on a DuPont Model 900 instrument at a scan rate of 10°C/minute exhibits a single sharp melt endotherm with an onset temperature of about 177°C and an extrapolated melting point of about 190°C followed by decomposition (15). A typical thermogram is shown in Figure 6.

#### 2.9 Thermogravimetric Analysis:

Thermogravimetric analysis of tripelennamine hydrochloride in a nitrogen atmosphere was obtained on a Perkin-Elmer TGS-1 Thermobalance. At a scan rate of 10°/min., a 3 mg sample showed a weight loss of 0.2% from room temperature to 155°C, followed by a rapid weight loss above 155°C (15).

#### 2.10 X-Ray Powder Diffraction:

The x-ray powder diffraction pattern of USP tripelennamine hydrochloride obtained on a Diano Model 8535 diffractometer using the Cu K $\alpha$  line (1.542 $\text{\AA}$ ) as the radiation source with a Ni filter is shown in Figure 7. Strong lines are observed at 12.6, 16.8, 19.0, 20.4, 21.2 and 21.9 degrees 2 $\theta$  under these conditions (15).

#### 2.11 Microscopy:

Tripelennamine hydrochloride USP reference standard is composed of tablet shaped birefringent crystals, as well as crystal fragments and aggregates. Microscopic crystallographic properties of tripelennamine hydrochloride have been reported in the literature and are summarized in Table VII. For both cases presented the samples were recrystallized from water.

Figure 6  
Differential Scanning Calorimetry Scan  
of Tripeleannamine Hydrochloride

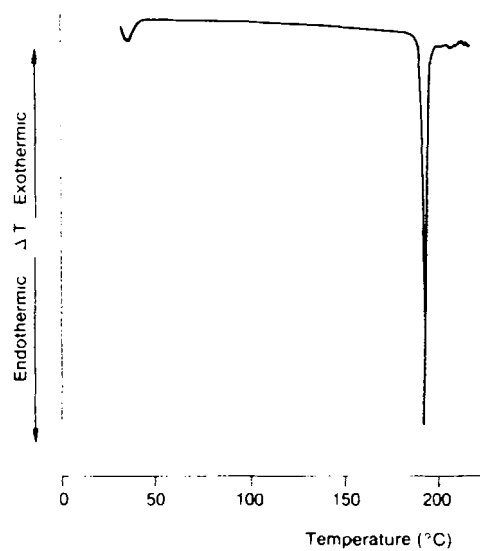


Figure 7  
X-ray Powder Diffraction Pattern of TripeleNNamine Hydrochloride

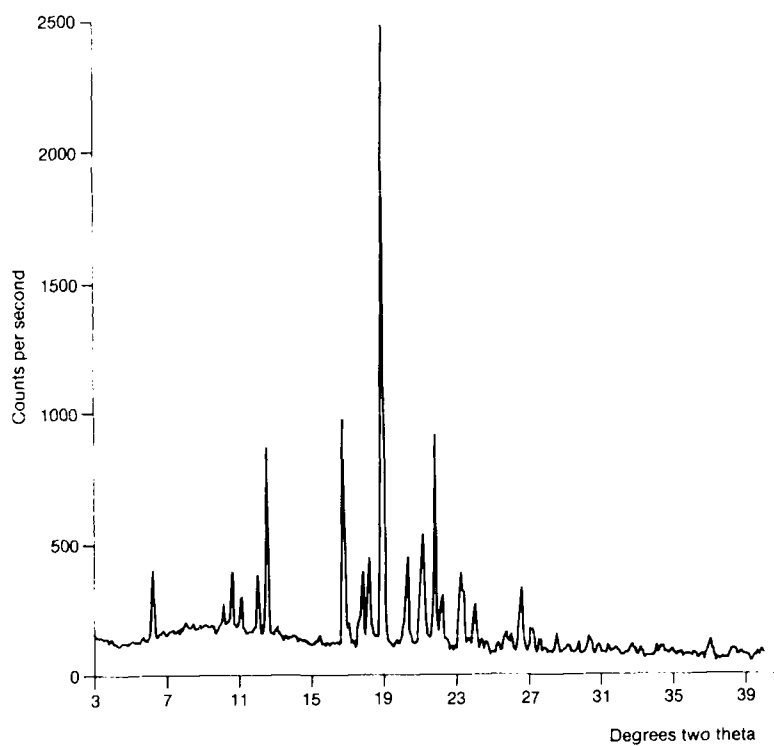




Table VIIMicroscopic Crystallographic Properties of Tripeleennamine Hydrochloride

Crystal System	N $\alpha$	N $\beta$	N $\gamma$	Optic Sign	2V	Habit
Monoclinic (16)	1.545	1.639	1.783*	+	84°	Tabular
Not Classified (17)	1.580	1.655	1.705	-		Rectangular plates and prisms

\*Calculated

Treatment of glycerol-alcohol (1 + 1) or water solutions of tripeleennamine hydrochloride with platinic chloride produces small needles and bladed crystals in dense rosette aggregates and singly (18,19).

2.12 Dissociation Constant:

Martindale's Pharmacopoeia reports dissociation constants of 3.9 and 9.0 for tripeleennamine at 25° (20), while the Merck Index gives a pK<sub>b</sub> for the free base of 4.93 (2).

Testa and Murset-Rossetti (21) reported pK<sub>a</sub> values  $8.68 \pm 0.06$  and  $3.90 \pm 0.08$  in water using the nonlogarithmic equations of Benet and Goyan (22).

2.13 Solubility:

The Merck Index (2) reports the following approximate solubilities for tripeleennamine hydrochloride. One gram of material dissolves in 0.77 ml water, in 6 ml alcohol, in 6 ml chloroform and in about 350 ml acetone. It is practically insoluble in benzene, ether and ethyl acetate.

2.14 Partition Coefficients:

The partition coefficients of tripeleennamine hydrochloride were determined at ambient conditions (~24°C) after shaking equal volumes of co-saturated solutions for chloroform/0.1N hydrochloric acid and chloroform/0.1N sodium hydroxide. The partition coefficient is defined as the concentration in the organic layer/concentration in the aqueous layer.

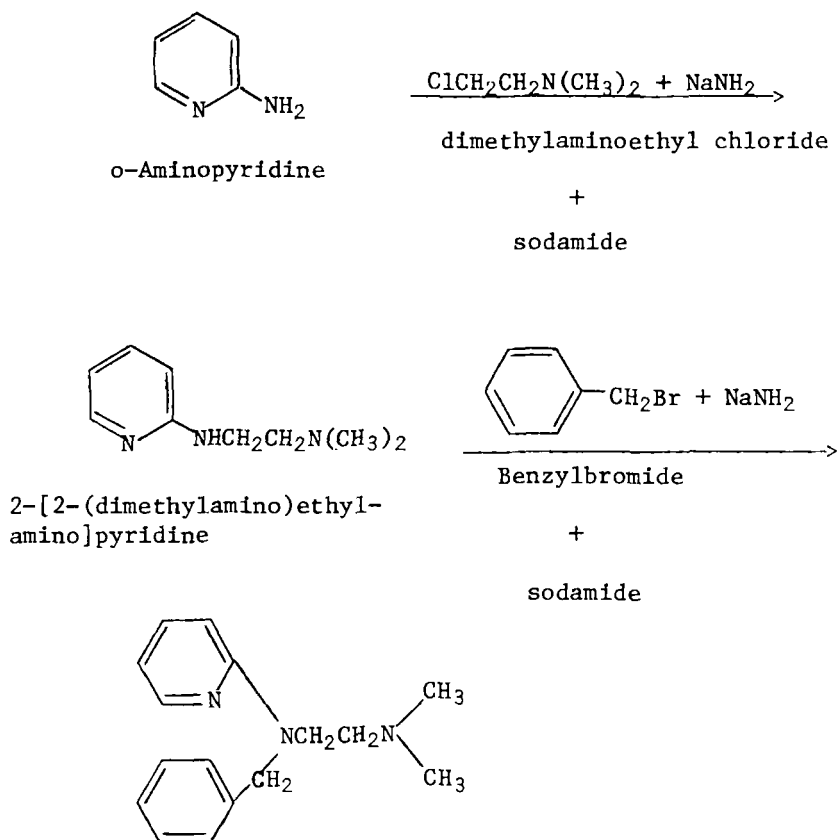
Table VIII

Organic Phase	Aqueous Phase	Partition Coefficient	Reference
Chloroform	0.1N HCl	0.004	23
Chloroform	0.1N NaOH	$\rightarrow \infty$	23

Testa and Murset-Rossetti (21) studied the octanol/water partition coefficient ( $P+$ ) of nineteen monoprotinated antihistaminic drugs. They report a log  $P+$  of -0.23 for tripelennamine.

### 3. Synthesis:

Tripelennamine can be synthesized by the following method:



The free base is converted to the hydrochloride by treatment with hydrogen chloride in an organic solvent (24,25).

#### 4. Stability:

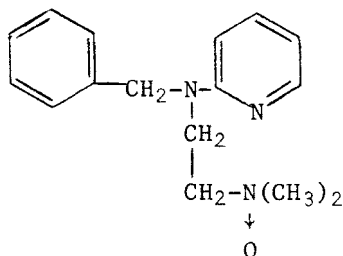
Tripelennamine hydrochloride is stable for at least 5 years when stored at room temperature, when protected from light. However, it slowly darkens when exposed to light (3) and appears to undergo light-catalyzed oxidation to yield benzaldehyde and 2-[2-(dimethylamino)ethylamino]pyridine.

Rao and Krishna (26) reported that tripelennamine reacts with sodium nitrite under physiological conditions to produce N-nitrosodesmethyltripelennamine.

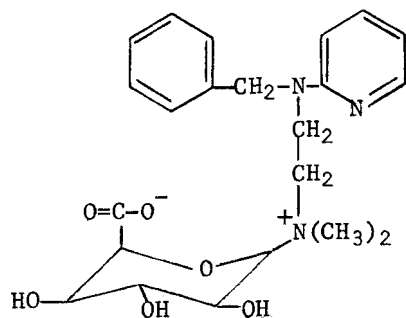
#### 5. Pharmacokinetics and Metabolism:

Studies with  $^{14}\text{C}$ -labeled tripelennamine in the rat (27) and guinea pig (28) indicate that the drug is rapidly absorbed from the gastrointestinal tract and localized in the tissues. The majority of tripelennamine radioactivity is eliminated in the urine within 24 hours. Tripelennamine seems to be metabolized by N-demethylation and aromatic hydroxylation followed by conjugation with glucuronic acid. Way and Dailey (29) concluded that tripelennamine is almost completely metabolized in the rat and that the liver is the most active organ in this metabolization.

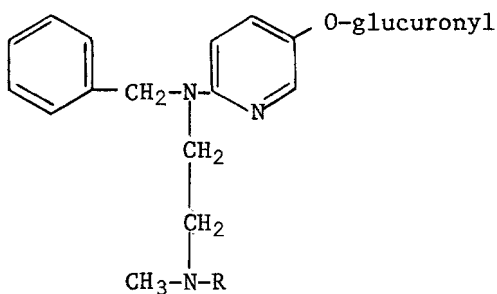
Early studies by Perlman (10) indicated that very little unchanged tripelennamine was excreted in human urine. When the urine was heated with alkali, however, 10% of the orally administered tripelennamine could be recovered from the urine. Chaudhuri and co-workers (30) isolated four polar metabolites from human urine. The structures of these metabolites are shown below.



N-oxide of tripelennamine



Quaternary ammonium N-glucuronide of tripeleennamine



R = CH<sub>3</sub>; O-glucuronide of hydroxytripeleennamine

R = H; O-glucuronide of hydroxydesmethyltripeleennamine

## 6. Analytical Methods:

### 6.1 Elemental Analysis:

The following elemental analysis was obtained on a sample of tripeleennamine hydrochloride on a Perkin-Elmer Model 240 CHN Analyzer.

<u>Element</u>	<u>Theory, %</u>	<u>Found, %</u>
Carbon	65.85	66.02
Hydrogen	7.60	7.62
Nitrogen	14.40	14.24

## 6.2 Nonaqueous Titration:

Tripeleennamine hydrochloride can be titrated in glacial acetic acid which contains mercuric acetate using 0.1N perchloric acid in glacial acetic as the titrant. The endpoint may be determined potentiometrically using a glass indicating electrode and a modified calomel reference electrode containing a saturated solution of lithium perchlorate in glacial acetic. Alternately, the endpoint may be detected visually using crystal violet as the indicator.

## 6.3 Phase Solubility Analysis:

Phase solubility analysis of tripeleennamine hydrochloride has been performed using isopropanol as the solvent. The approximate solubility of the compound is 16.4 mg/gm of solvent.

## 6.4 Thin-Layer Chromatography:

Several thin-layer chromatographic systems have been employed for the identification and the determination of tripeleennamine hydrochloride and related compounds.

System I: This system can be used to monitor the content of the starting material, o-aminopyridine. Silica Gel GF<sub>254</sub> (Fisher); chloroform/diethylamine (9:1); ultraviolet light (<sub>254</sub>) detection.

System II (31): The following system has been employed to detect tripeleennamine in urine when screening for drug abuse. Silica Gel G/UV<sub>254</sub> (Brinkman); hexane/ethyl acetate/ethanol/concentrated ammonium hydroxide (45/50/5/2); iodoplatinate detection.

System III (32): The following system has also been used in identification of tripeleennamine in drug abuse urine screening programs. Silica gel glass microfiber sheets (Gelman ITLC Type SA); ethyl acetate/cyclohexane/methanol/concentrated ammonium hydroxide (56/40/0.8/0.4); iodoplatinate detection.

## 6.5 Gas Chromatography:

The following gas chromatographic systems have been used to determine tripeleennamine hydrochloride.

System I (33): The following method is used to control the active ingredient alone and in pharmaceutical formulations. This method separates the photolytic decomposition products, benzaldehyde and 2-[2-dimethylamino)ethylamino]-pyridine.

Column: 4 ft. x 4 mm (I.D.) glass column containing 2.5% UCW 98 on 80-100 Gas Chrom Q

Temperature: Hewlett Packard Model 7620A Chromatograph; injector (250°C), column (200°C), detector (250°C)

Carrier: Helium at 25 ml/minute

Detection: Flame ionization detection of underivatized drug.

System II (34): The following procedure has been used to determine tripeleennamine hydrochloride residues in bovine milk.

Column: 8 ft. x 4 mm (I.D.) Borosilicate column packed with 2% Carbowax 20M on 100-200 mesh Gas-Chrom S, previously washed with base.

Temperature: Injector (230°C), column (218°C), detector (240°C)

Carrier: Nitrogen at 60 ml/min.

Detection: Flame ionization detection of underivatized tripeleennamine.

System III (35,36): The following system was used to quantitate the concentration of tripeleennamine hydrochloride in the blood of pentazocine and tripeleennamine addicts.

Column: 1.8 m x 4 mm (I.D.) glass column packed with 3% OV-17 on 80-100 mesh Chromosorb W HP

Temperature: Injector (250°C), column (220°C), detector (275°C)

Carrier: Nitrogen at 30 ml/min.

Detection: Nitrogen detector for underivatized drug.

System IV (31): The following system has been employed for identification of tripeleennamine in urine.

Column: 1.8 m x 2 mm (I.D.) glass column  
packed with 3% OV-17 on 80-100 mesh  
Gas-Chrom Q

Temperatures: Injector (250°C), column (230°C),  
detector (270°C)

Carrier: Helium at 30 ml/min.

Detector: Flame ionization of underivatized  
tripeleennamine

In addition, data for tripeleennamine hydrochloride have been reported in the literature as part of procedures for general classes of compounds. MacDonald and Pflaum (37) investigated the gas chromatographic behavior of sixteen antihistamines on Carbowax 20M, SE-30, XF-1150 and PDEAS. Fabregas (38) employed 3% SE-30 on Chromosorb 80-100 W HP to assay traces of tripeleennamine in pharmaceutical preparations. General methods of drug screening have been reported which include data for tripeleennamine (39,40).

#### 6.6 Gas Chromatography - Mass Spectrometry:

The method reported below has been used to identify tripeleennamine in urine (31). The mass spectrometric work was carried out in the chemical ionization mode.

Column: 1.8 m x 2 mm (I.D.) glass column  
packed with 2% OV-17 on 100-200  
mesh Gas-Chrom Q

Temperatures: Column (programmed from 150°C  
to 250°C, at 10°C/min.), separator  
(250°C), transfer line (240°C),  
manifold (72°C)

Carrier and  
Ionization Gas: Methane (30 ml/min through  
column)

Sample: Underivatized tripeleennamine

### 6.7 High Pressure Liquid Chromatography:

The following system has been used in the separation of various antihistamine drugs (41).

Column:	Mikropak-CN-10
Mobile Phase:	1) N-Heptane/methylene chloride/ acetonitrile/propylamine (50/50/25/0.1)
	2) methanol/water/propylamine (90/10/0.01)
	3) acetonitrile/water/propyl- amine (90/10/0.01)
Flow Rate:	80 ml/hr for mobile phase 1
	30 ml/hr for mobile phases 2 and 3
Detection:	Ultraviolet absorption (254 nm)

### 6.8 Column Chromatography:

Blaug and Zopf (42) reported the separation of antihistamines using basic Amberlite ion exchange resins. In addition, separation of tripeleennamine hydrochloride from ephedrine in an elixir preparation was carried out on Amberlite IRC-50, a carboxylic cation exchanger. The separated antihistamines were then quantitated using titration with 0.1N hydrochloric acid.

### 6.9 Ultraviolet Spectrometry:

The assay procedure and content uniformity determination in the USP XX (5) monograph for tripeleennamine hydrochloride tablets employs ultraviolet absorption after a back and forth extraction described for the determination of salts of organic nitrogenous bases.

### 6.10 Colorimetric:

#### 6.10.1 Cyanogen Bromide:

When tripeleennamine hydrochloride is treated with cyanogen bromide in potassium acid phthalate solution, followed by the addition of aniline a yellow colored product is formed (43). The drug may be quantitated by measuring the absorbance at 412 nm.



#### 6.10.2 Reineckate Salt

Tripelennamine hydrochloride is reacted with ammonium reineckate,  $\text{NH}_4[\text{Cr}(\text{NH}_3)_2(\text{SCN})_4]$ , in aqueous solution producing a precipitate which is dissolved in acetone and the absorbance determined at 525 nm (44).

#### Acknowledgement:

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For this profile the literature has been searched through Chemical Abstracts 1946.

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# XYLOMETAZOLINE HYDROCHLORIDE

Yechiel Golander and Wayne J. DeWitte

*CIBA-GEIGY Corporation  
Suffern, New York*

1. Description	136
1.1 Nomenclature	136
1.2 Formula	136
1.3 Molecular Weight	136
1.4 Elemental Composition	136
1.5 Appearance, Color, and Odor	137
1.6 Patent Information/Literature	137
2. Physical Properties	137
2.1 Infrared Spectrum	137
2.2 Nuclear Magnetic Resonance Spectrum	137
2.3 Ultraviolet Spectra	137
2.4 Mass Spectrum	137
2.5 Melting Range	144
2.6 Thermogravimetric Analysis (TGA)	144
2.7 Differential Scanning Calorimetry (DSC)	144
2.8 Microscopy	144
2.9 Polymorphism	144
2.10 X-Ray Powder Diffraction	144
2.11 Density and Bulk Volume	146
2.12 Dissociation Constant	146
2.13 Partition Coefficient	146
2.14 Solubility	146
3. Synthesis	146
4. By-Products, Stability, and Degradation	148
4.1 By-products	148
4.2 Degradation Reactions and Stability	148
5. Pharmacology	150
6. Methods of Analysis	150
6.1 Elemental Analysis	150
6.2 Volumetric Methods	151
6.3 Colorimetric Methods	151
6.4 Liquid Chromatographic Methods	152
6.5 Gas Chromatographic Methods	152
6.6 Thin-Layer Chromatographic Methods	153
7. Identification and Determination in Dosage Forms	153
7.1 Identification	153
7.2 Determination	153
References	155

1. Description:1.1 Nomenclature:1.1.1 Chemical Names:

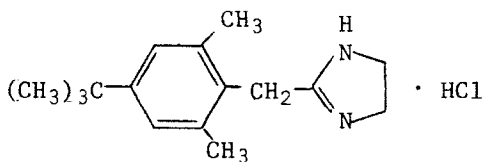
- a) 1H-Imidazole, 2-[[4-(1,1-dimethylethyl)-2,6-dimethylphenyl]-methyl]-4,5 dihydro-monohydrochloride.
- b) 2-(4-tert-butyl-2,6-dimethylbenzyl)-2-imidazoline monohydrochloride.
- c) 2-[[4-(1,1-Dimethylethyl)-2,6-di-methyl-phenyl]methyl]-4,5-dihydro-1H-imidazole hydrochloride

1.1.2 Generic Name:

Xylometazoline hydrochloride

1.1.3 Proprietary Names:

Otrivin, Otriven, Novorin, Otrix

1.2 Formula:1.2.1 Empirical:  $C_{16}H_{24}N_2 \cdot HCl$ 1.2.2 Structural:1.2.3 CAS No.: [1218-35-5]; free base [526-36-3]1.3 Molecular Weight:

280.84

1.4 Elemental Composition:

C, 68.43%; H, 8.19%; N, 9.97%; Cl, 12.62%

### 1.5 Appearance, Color and Odor:

White, crystalline, odorless substance.

### 1.6 Patent Information/Literature:

U.S. Pat. 2,868,802 (1959 to CIBA)

## 2. Physical Properties:

### 2.1 Infrared Spectrum:

The infrared spectrum (Figure 1) was obtained from a mineral oil dispersion of xylometazoline hydrochloride (1) using a Perkin-Elmer Model 281B IR spectrophotometer. The assignments for important absorption bands are presented in Table I (2).

### 2.2 Nuclear Magnetic Resonance Spectrum:

The NMR spectrum (Figure 2) of xylometazoline hydrochloride was obtained in  $\text{CDCl}_3$  containing TMS as internal reference, using a JEOL FX90Q NMR spectrometer. The spectrum was run at 90 MHz and ambient temperature. Spectral assignments of the signals are given in Table II (2,3).

### 2.3 Ultraviolet Spectra:

Typical ultraviolet spectra of xylometazoline hydrochloride in water and 0.1N HCl using a Hewlett-Packard 8450A UV/VIS spectrophotometer are shown in Figure 3. Padmanabhan (4) reported the ultraviolet absorption spectrum for xylometazoline hydrochloride in water to show a shoulder at 217 nm with  $A(1\%, 1\text{cm}) = 546$ .

### 2.4 Mass Spectrum:

The mass spectrum for xylometazoline hydrochloride (Figure 4) was obtained with a Kratos Model MS-25 mass spectrometer. The m/e assignments are presented in Table III (2,5).



Figure 1  
A Typical IR Spectrum of Xylometazoline Hydrochloride In Nujol

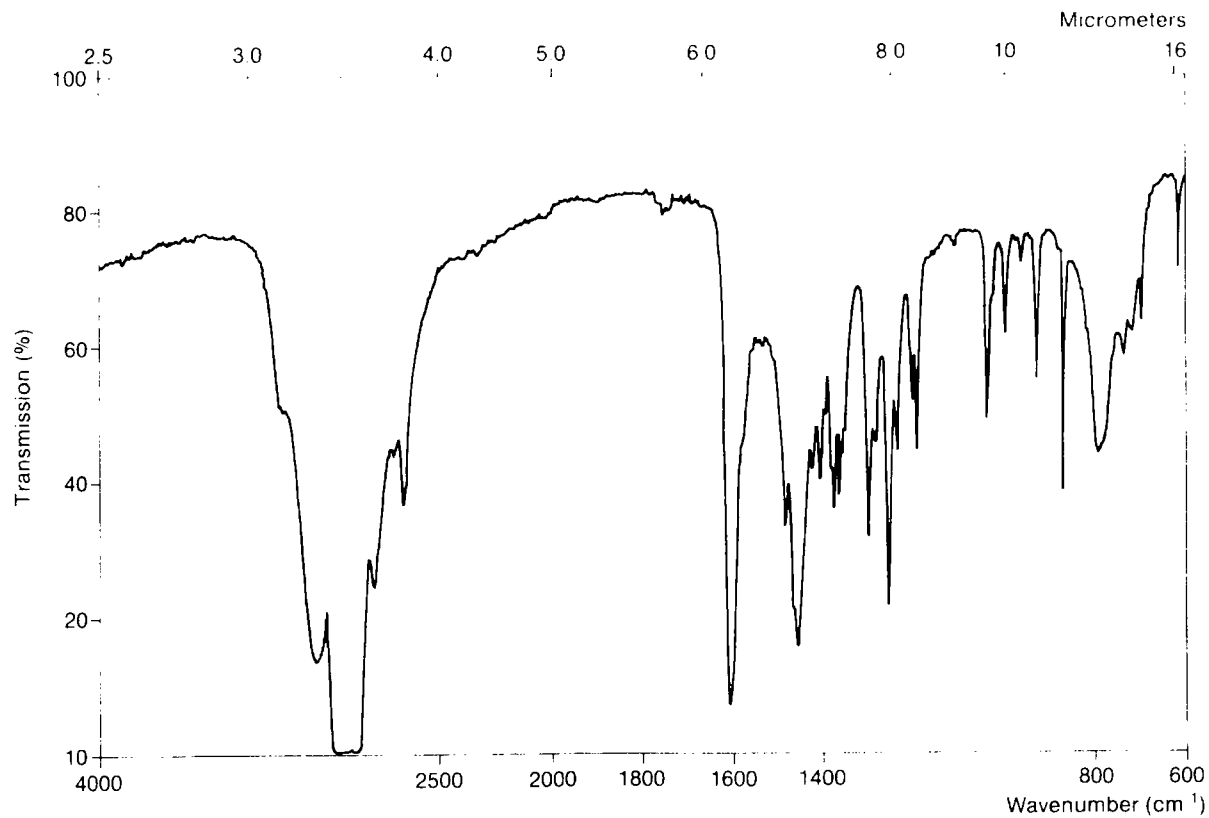


TABLE I

Infrared Assignments for Xylometazoline Hydrochloride

<u>Wavenumber (cm<sup>-1</sup>)</u>	<u>Assignment</u>
3300 - 2600	⊕ NH stretching, CH stretching and nujol
1610	imidazoline C=N stretching
1490	aromatic C=C stretching
1210, 1195	<u>t</u> -Butyl skeletal vibration
875	aromatic CH bending
800	⊕ NH bending

TABLE II

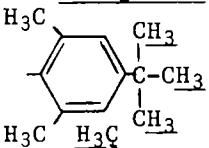
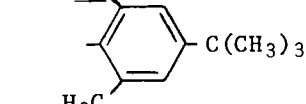
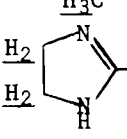
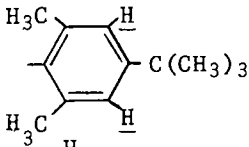
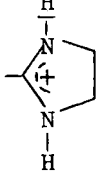
<u>δ (ppm)</u>	<u>Multiplicity</u>	<u>No. of Protons</u>	<u>Assignment</u>
1.26	Singlet	9	
2.30	Singlet	6	
3.90	Singlet	4	
4.10	Singlet	2	-CH <sub>2</sub> -
7.06	Singlet	2	
9.5	Singlet (broad)	2	

Figure 2  
A Typical NMR Spectrum of Xylometazoline Hydrochloride in CDCl<sub>3</sub>

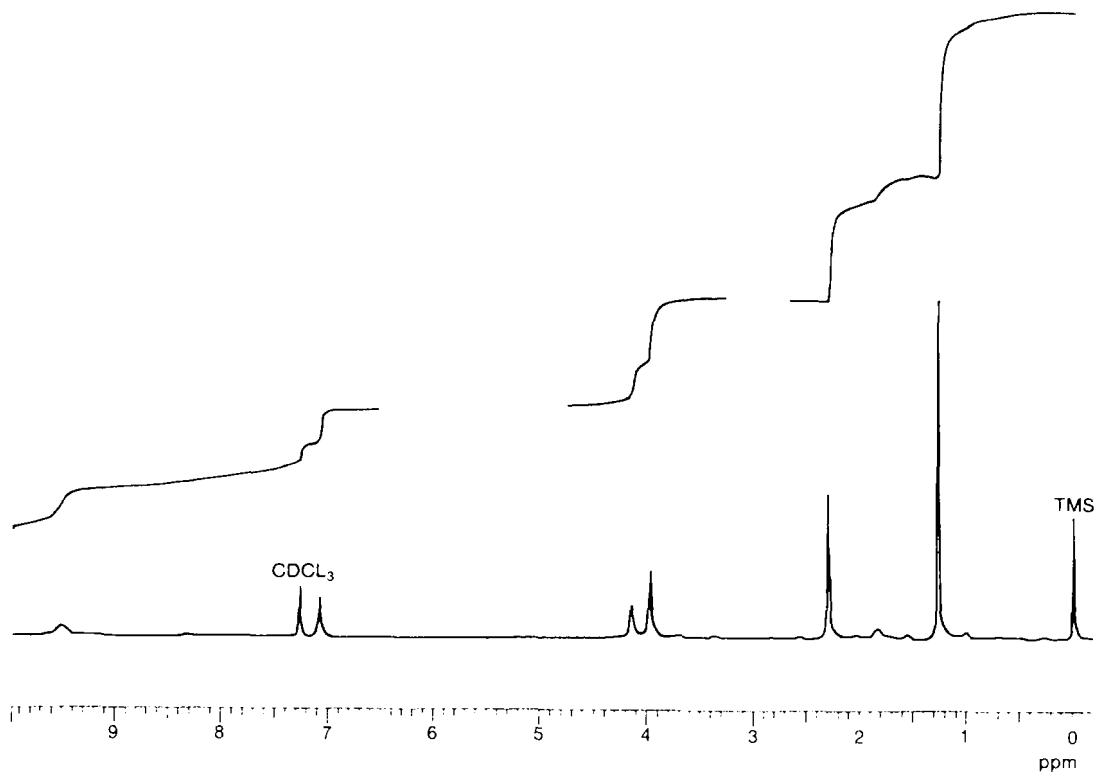


Figure 3  
Typical UV Spectra of Xylometazoline Hydrochloride  
in Water and 0.1N HCl

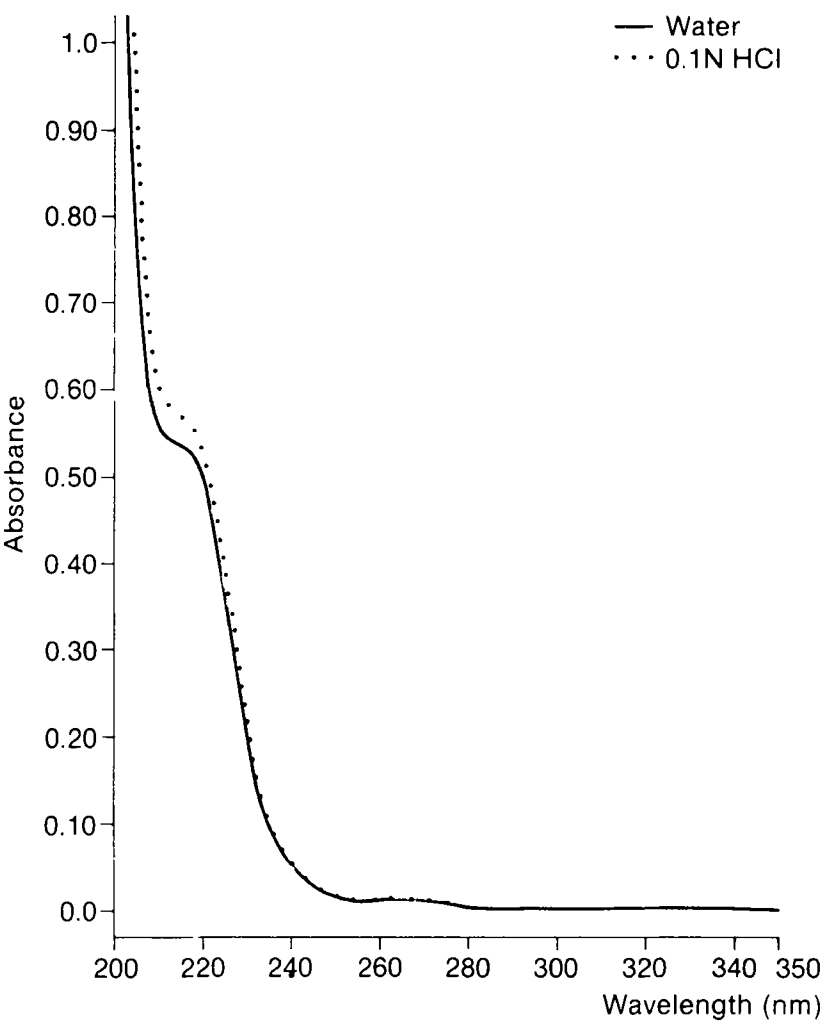


Figure 4  
A Typical EI Mass Spectrum of Xylometazoline Hydrochloride

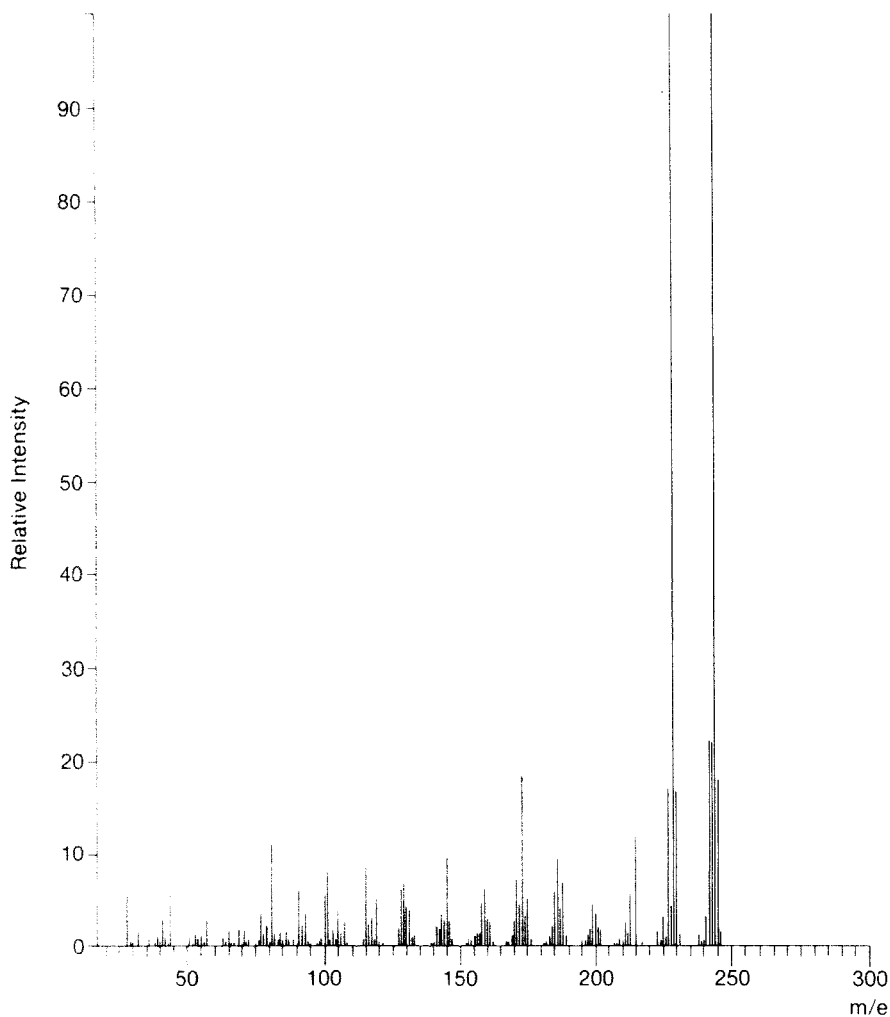
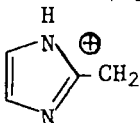


TABLE III

Mass Spectral Assignments for Xylometazoline\*

<u>m/e</u>	<u>Relative Intensity (%)</u>	<u>Assignment</u>
245	17.8	$(M + 1)^+$
244	99.9	$M^+$
243	21.6	$(M - 1)^+$
242	21.9	$(M - 2)^+$
229	100.0	$(M - CH_3)^+$
227	16.8	$(242 - CH_3)^+$
215	12.0	$(242 - HCN)^+$
173	18.2	$(229 - C_4H_8)^+$
81	11.1	

\*Hydrochlorides of organic amines dissociate into the free base and hydrochloric acid on evaporation in the mass spectrometer (2).

## 2.5 Melting Range:

The melting range for xylometazoline free base is 131-133°C (6). The hydrochloride salt has a melting range of 317-324°C with decomposition in air. Decomposition does not occur in a nitrogen atmosphere (7).

## 2.6 Thermogravimetric Analysis (TGA):

The thermogravimetric behavior of xylometazoline hydrochloride was determined in a N<sub>2</sub> atmosphere at a scan rate of 10°C/minute (8). No weight loss was observed from 25°-60°C. From 60°C-180°C a 0.14% weight loss was observed, and above 180°C the weight loss became rapid, due to sublimation (7).

## 2.7 Differential Scanning Calorimetry (DSC):

Under nitrogen atmosphere, xylometazoline hydrochloride had an observed melting point of 332.3°C, with a latent heat of fusion of 48.8 kJ/Mol (7).

## 2.8 Microscopy:

Xylometazoline hydrochloride powder is composed of irregular shaped crystals and crystal fragments which exhibit extinction and birefringence under crossed polars (8).

## 2.9 Polymorphism:

Only one crystal form is known for xylometazoline hydrochloride (7).

## 2.10 X-ray Powder Diffraction:

The X-ray powder diffraction pattern of xylometazoline hydrochloride is presented in Table IV. Strong lines are observed at interplanar distances ( $d = n\lambda/2 \sin \theta$ ) of 3.74, 6.06 and 5.24 Å. The X-ray diffraction photographs were obtained using a 114.83 mm diameter powder camera, using copper K $\alpha$  radiation with a Ni filter. The X-ray powder diffractometer patterns were recorded by mounting ~2g of ground sample into an aluminum specimen holder and exposing it to the X-ray beam for 45 minutes (9).

TABLE IV

X-ray Powder Diffraction Pattern of Xylometazoline  
Hydrochloride\*

<u>d, (Å)**</u>	<u>Relative Intensity (%)***</u>
13.90	55
7.43	54
7.03	8
6.06	89
5.24	76
5.02	6
4.65	19
4.44	68
4.34(s)	26
4.27	37
4.04	38
3.95	31
3.87	14
3.74	100
3.52	24
3.46	19
3.28	25
3.10	25
3.02	15
2.89	12
2.81	11
2.71	8
2.62	8
2.49	6
2.34	9

\*From Reference 9

\*\*Interplanar Distance:  $d = n\lambda / 2 \sin \theta$

\*\*\*Relative Intensity in percent based on strongest signal.

(s) Shoulder or peak poorly resolved from stronger signals.



### 2.11 Density and Bulk Volume:

The density of a typical sample of xylometazoline hydrochloride has been reported as 1.14 g/cm<sup>3</sup> (10).

### 2.12 Dissociation Constant:

The pKa for xylometazoline hydrochloride has been determined, by photometric titration in water at 22°C, to be 10.6 ± 0.1 (11).

### 2.13 Partition Coefficient:

The partition coefficient of xylometazoline hydrochloride was determined to be 2.34 for the n-octanol/phosphate buffer pH 7.4 system at 25°C (11). The coefficient is defined as K<sub>p</sub>, where:

$$K_p = \frac{S(\text{organic})}{S(\text{aqueous})}$$

and S is the concentration of xylometazoline hydrochloride in each phase.

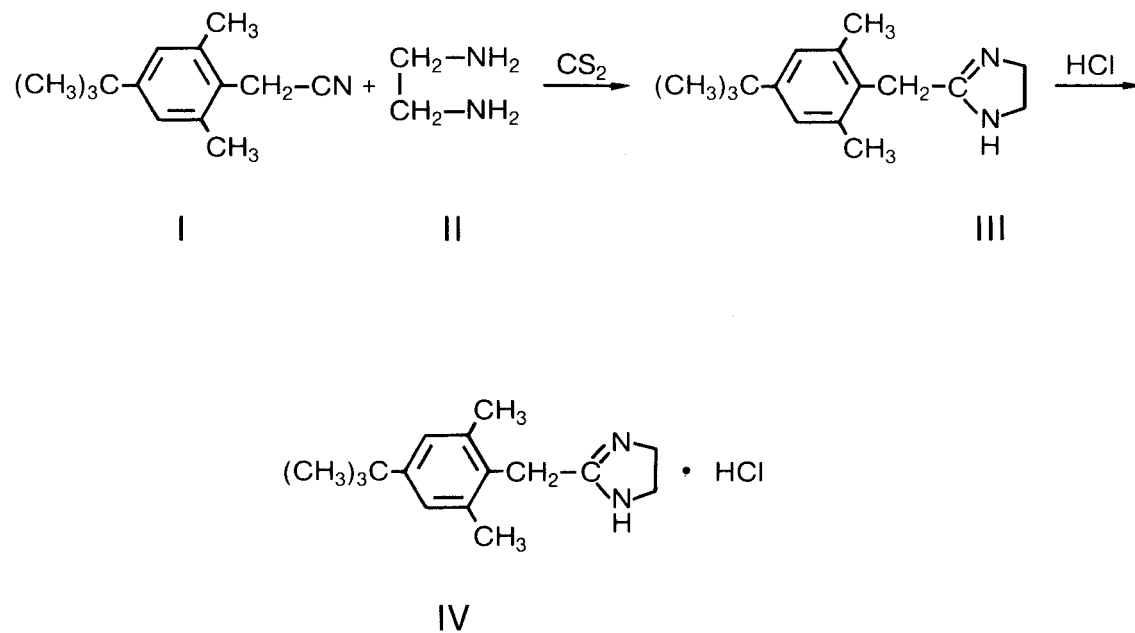
### 2.14 Solubility:

The solubility of xylometazoline hydrochloride is reported to be 3% in water (6). Xylometazoline hydrochloride is also soluble in methanol and ethanol, and practically insoluble in ether and benzene (6). The solubility in water has also been reported as 1 g in 35 ml at 25°C (12).

## 3. Synthesis:

The chemical reaction sequence reported for the synthesis of xylometazoline hydrochloride (11,13) is shown in Figure 5. 2,6-Dimethyl-4-tert-butyl-benzylcyanide (I) is converted into the corresponding imidazoline (III) by heating with ethylenediamine (II) in the presence of carbon disulfide as catalyst. The imidazoline-base (III) is purified by recrystallization from heptane. The purified base is then dissolved in methyl-ethyl ketone and the hydrochloride (IV) is produced by passing hydrogen chloride gas through the solution. The hydrochloride (IV) is dissolved in absolute alcohol and allowed to crystallize.

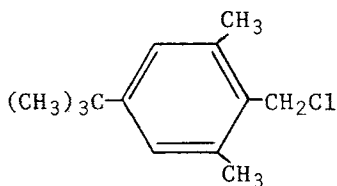
Figure 5  
The Synthetic Pathway of Xylometazoline Hydrochloride



#### 4. By-Products, Stability and Degradation:

##### 4.1 By-Products:

Potential by-products originating from the manufacturing process are the components of the last steps of the synthesis of xylometazoline (11), i.e. 2,6-dimethyl-4-tert-butyl-benzylcyanide (I), ethylenediamine (II) [see Figures 5 and 6] and the precursor of (I), 2,6-dimethyl-4-tert-butyl-benzylchloride (V):



(V)

The presence of organic sulfur compounds, coming from the use of carbon disulfide as a condensation catalyst in the final step of the synthesis, is also possible.

Small quantities of hydrolysis products VI and VII (see Figure 6) may be formed during the synthesis of xylometazoline hydrochloride. These are further discussed under "Degradation Reactions and Stability" (§4.2).

##### 4.2 Degradation Reactions and Stability:

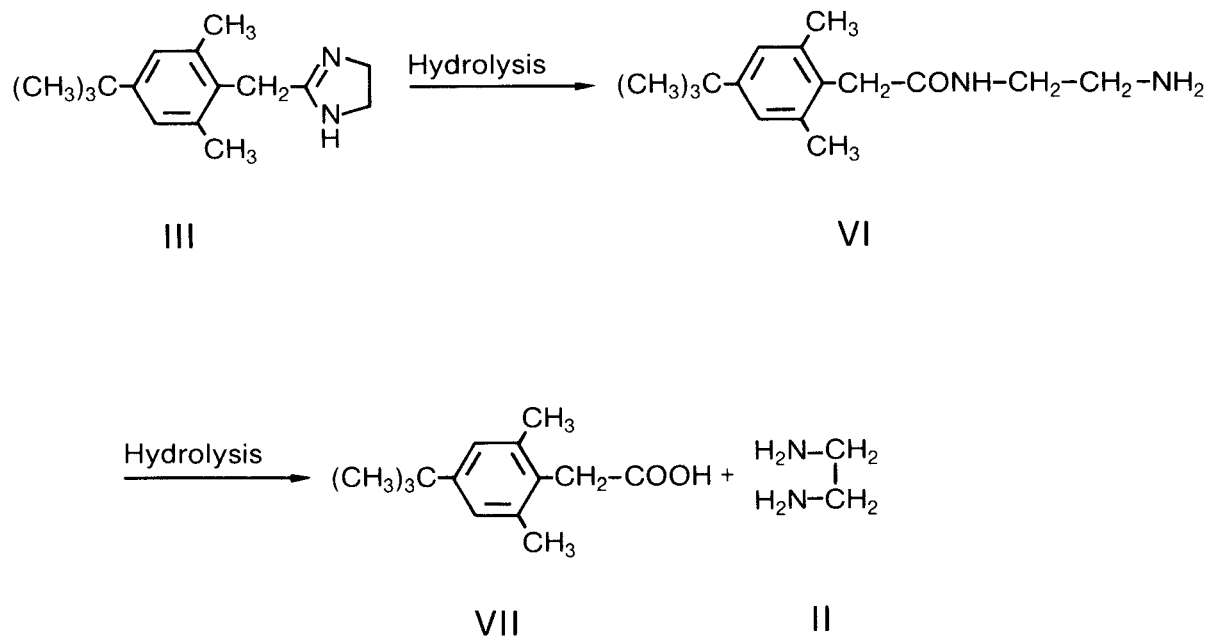
###### 4.2.1 Solid State Stability:

In the absence of light and moisture, xylometazoline hydrochloride is stable for at least 8 years at room temperature (14).

###### 4.2.2 Stability in Solution:

As a derivative of 2-imidazoline, xylometazoline hydrochloride may undergo the typical amidine hydrolysis reaction in aqueous solutions, where 2,6-dimethyl-4-tert-butyl-phenylacetyl ethylenediamine (VI) is first formed (11,15) [Figure 6]. Under more severe hydrolytic conditions the amide (VI) yields 2,6-dimethyl-4-tert-butyl-phenylacetic acid (VII) and ethylenediamine (II). The course of hydrolysis is strongly influenced by temperature and pH. Xylometazoline

Figure 6  
The Hydrolysis Products of Xylometazoline Hydrochloride



hydrochloride is fairly stable in acid or neutral media, whereas in alkaline media the rate of hydrolysis is considerably increased (15). Thus, three decomposition products of xylomethazoline have been separated and isolated from the alkaline reaction mixture, and were identified as Compounds II, VI and VII. No decomposition products were obtained from a reaction mixture of xylomethazoline at pH 1 (0.1N HCl). However, xylometazoline hydrochloride was found to be more resistant to hydrolysis than other imidazoline derivatives such as naphazoline, tolzaoline and antazoline (16). Even when the alkaline conditions reported by Schwartz and others (17) were applied [0.5N NaOH; reflux 30 min.], no hydrolysis occurred. This can be ascribed to steric hindrance from the two ortho methyl groups.

A 0.1% aqueous solution of xylometazoline hydrochloride in the presence of 0.02% benzalkonium chloride at pH 6.3 is stable at room temperature, 6°C, 35°C and 45°C for at least 4 years (18).

## 5. Pharmacology:

Xylometazoline hydrochloride is a sympathomimetic agent with marked alpha-adrenergic activity. It constricts the smaller arterioles of the nasal passages, effecting a decongesting action. It is used in 0.1% and 0.05% solutions for the relief of nasal congestion caused by rhinitis and sinusitis. A 0.05% solution may be instilled into the eye as a conjunctival decongestant. The pharmacological properties of xylometazoline have been studied by Morimoto and Tanaka (19).

## 6. Methods of Analysis:

### 6.1 Elemental Analysis:

The results from an elemental analysis of xylometazoline hydrochloride are listed below (20):

#### Elemental Analysis of Xylometazoline Hydrochloride

<u>Element</u>	<u>% Theory</u>	<u>% Found</u>
Carbon	68.43	68.69
Hydrogen	8.97	8.68
Nitrogen	9.97	9.85

## 6.2 Volumetric Methods:

Xylometazoline hydrochloride USP is assayed by titration with perchloric acid in glacial acetic acid, adding mercuric acetate and p-naphtholbenzein as indicator (1). The chloride content of the hydrochloride salt may also be determined by argentometric titration.

## 6.3 Colorimetric Methods:

Several colorimetric techniques for determination of imidazoline drugs, including xylometazoline, are reported. The official assay method for determination of xylometazoline hydrochloride in the nasal solution dosage form is reported in USP XX (1). The free base is extracted into dichloromethane, evaporated to dryness, redissolved in ethanol, made alkaline and reacted with sodium nitroferricyanide. The color thus developed is measured at 565 nm. This method is a modification of the procedure reported by Slack and Mader (21).

The reaction of the imidazoline ring in xylometazoline hydrochloride with sodium nitroferricyanide has also been used in a stability-indicating assay. In this report (22), xylometazoline hydrochloride is determined in the presence of its hydrolysis product, 2,6-dimethyl-4-tert-butyl-phenylacetyl ethylenediamine hydrochloride, by reaction with sodium nitroferricyanide. Subsequently, the color forming reaction of both xylometazoline and the decomposition product with tropeoline peroxide allows determination (at 410 nm) of both species simultaneously. The hydrolysis product content is then determined by difference. Alternatively, xylometazoline hydrochloride and its decomposition product may be first separated by thin-layer chromatography and determined separately (22).

Xylometazoline hydrochloride may be determined colorimetrically following complexation with dithizone (23). The sample is made basic, in the presence of KCN, and is treated with a chloroform solution of dithizone. The chloroform phase containing the complex is measured at 490 nm.

Several imidazoline drugs, including xylometazoline hydrochloride, have been determined colorimetrically following complexation with indicator dyes. The drug is reacted with the dye and then extracted into chloroform. Measurements of absorbance are made at 415 nm for complexes of xylometazoline

hydrochloride with bromcresol green, bromothymol blue or methyl orange, and at 420 nm for the complex with bromophenol blue (24).

#### 6.4 Liquid Chromatographic Methods:

Xylometazoline has been assayed successfully via high performance liquid chromatographic methods. Normal phase chromatography can be used to separate nitrogenous drugs, including xylometazoline, using a silica gel column and a mobile phase composed of dichloromethane, methanol and ammonium hydroxide (25). Another reported method uses the ion exchange mechanism to separate xylometazoline hydrochloride (drug substance or dosage form) from its hydrolysis product and/or from other imidazoline drugs (26).

#### 6.5 Gas Chromatographic Methods:

Xylometazoline hydrochloride can be analyzed via gas chromatography after conversion to the free base and extraction into dichloromethane. Xylometazoline was determined using flame ionization detection and columns packed with various Gaschrom supports coated with 3% OV-1, 3% OV-3, 3% OV-7, 2% OV-17 or 5% QF-1 stationary phases (27).

Imidazoline drugs have also been analyzed following isolation of free base in chloroform. Xylometazoline has been determined at the 0.05% level in imidazoline mixtures using a 4 ft. glass column containing 1% Carbowax 20M and 2% KOH on Gaschrom P (16).

Xylometazoline hydrochloride can be identified via a screening procedure utilizing combined thin-layer, gas and liquid chromatographic techniques. This procedure is used to screen about 570 drugs and organophosphorus pesticides rapidly for toxicological or pathological tests. The gas chromatographic systems for xylometazoline include OV-1 and OV-17 stationary phases, and the thin-layer chromatographic systems utilized silica gel plates with a) methanol-ammonia or methanol-hydrochloric acid and b) dichloromethane-acetone developing solvents (28). Detection for the thin-layer procedures was accomplished by spraying with a) Dragendorff reagent or b) exposure to  $\text{Cl}_2$  gas followed by spraying with 0.5% ethanolic o-tolidine, respectively.

## 6.6 Thin-Layer Chromatographic Methods:

In addition to the thin-layer screening procedure mentioned above (28), a second TLC screening procedure for a large number of drugs, including the imidazolines, is reported by Schmidt (29). This procedure employs three developing systems for silica gel plates, and twenty-six detection reagents.

A stability-indicating TLC procedure for xylometazoline hydrochloride and its principle hydrolysis product has been reported by Grabowski and Rajzer (15). Silica gel plates were developed with a mixture of benzene, acetone and ammonium hydroxide (3:25:3). Five detection reagents were used.

## 7. Identification and Determination in Dosage Forms:

### 7.1 Identification:

#### 7.1.1 Polarography:

Xylometazoline in nasal solutions is identified via the conversion of the free base into the N-nitroso derivative (30) and comparison of the half-wave potential to that of a similarly treated reference standard (31).

#### 7.1.2 Reaction with Sodium Nitroferricyanide:

A rose-violet color develops upon reacting a nasal solution of xylometazoline with sodium nitroferricyanide under alkaline conditions (31,32).

### 7.2 Determination:

#### 7.2.1 Colorimetric Method with Sodium Nitroferricyanide:

The USP assay for xylometazoline hydrochloride nasal solution (33) is a colorimetric stability-indicating method which was originally developed by S. Slack and W. Mader (21). This method consists of the free base extraction from the nasal solution and reaction with sodium nitroferricyanide in alkaline medium. The color formed by this reaction is measured at about 565 nm.

#### 7.2.2 Colorimetric Method with Cobaltous Acetate:

This method was originally developed by Bult and Klasen (34). The reagent cobaltous acetate forms a colored complex with the imidazoline functionality, which exhibits a  $\lambda_{\text{max}}$  at 565 nm. A 0.1% commercial nasal prepara-



tion of xylometazoline hydrochloride was assayed successfully using this procedure.

#### 7.2.3 Reaction with Dyes:

The reactions of several drugs, including xylometazoline hydrochloride, with three dyes (Solochrome Black T, Solochrome Dark Blue and Fast Sulphone Black FF) to form colored ion-pair complexes have been used to determine the drug content of various dosage forms (35).

#### 7.2.4 Gas Chromatographic Method:

A gas chromatographic method for solutions containing xylometazoline hydrochloride alone and in combination with other drugs was described by Boon and Sudds (16). The extracted free base is chromatographed on a glass column packed with Carbowax 20M (1%) + potassium hydroxide (2%) on acid and alkali washed Gaschrom P (100-140 mesh). The column is normally operated at 197°C with an argon flow rate of 40 ml/min.

#### 7.2.5 Liquid Chromatographic Methods:

A stability indicating method for xylometazoline hydrochloride nasal drops (0.1% solution), employs a Nucleosil 10 C<sub>18</sub> column and a mobile phase of water:acetonitrile (60:40) plus 0.25% ammonium sulfate. The flow rate is 3 ml/minute and the wavelength of detection is 220 nm (36).

Xylometazoline hydrochloride in liquid dosage form has been assayed following separation in a chromatographic column loaded with amberlite IR-120 ion exchange resin. The method is reported to be as accurate as the official compendial methods (37).

#### Acknowledgement:

The Authors express appreciation to Deborah Knortz and Richard Brown for help in preparation of this manuscript.

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# MEFLOQUINE HYDROCHLORIDE

Peter Lim

*SRI International  
Menlo Park, California*

1. Introduction	158
2. Description	158
2.1 Nomenclature	158
2.2 Formula	159
2.3 Appearance, Color, and Odor	160
3. Physico-chemical Properties	160
3.1 Elemental Analysis	160
3.2 Melting Characteristics	160
3.3 Spectral Characteristics	160
3.4 Solubility	166
3.5 Ionization Constant	170
4. Synthesis	170
4.1 Overall Preparation	170
4.2 <sup>14</sup> C-Labelled Preparation	171
4.3 Optical Isomer Preparation	172
5. Analytical Methods	172
5.1 Qualitative	172
5.2 Quantitative	174
6. Stability	176
6.1 Bulk	176
6.2 Solution	176
6.3 Dosage Form	177
7. Biopharmaceutics	177
7.1 Pharmacokinetics	177
7.2 Metabolism	178
7.3 Animal Toxicology	178
References	179

## 1. Introduction

In a recent medical progress review on malaria by C.J. Wiler (1), the author cited a quotation from "National History of Infectious Disease" by Sir Macfarlane Burnet. It reads, if we take as our standard of importance the greatest harm to the greatest number, then there is no question that malaria is the most important of all infectious diseases.

The recent resurgence of malaria has renewed human suffering on a staggering scale. The cause of the resurgence is complex and includes a number of social and biological factors. Ironically, one of these factors was the World Health Organization's success in eradicating malaria in 1960s by large scale spraying of insecticides. This success lulled malaria research into complacency and engendered a reduction in the efforts in the control and treatment of this wide-spread disease. Another decisive factor was the wide-spread resistance to DDT among several anophiles mosquito vectors, and many strains of *Plasmodium falciparum* became resistant to chloroquine and other antimalarials.

More than 95% of the malaria in the world is caused by two species of plasmodia that naturally infect humans. These species are *Plasmodium vivax* and *P. falciparum*. The disease caused by the latter is the more serious and is usually fatal in nonimmune persons if not treated promptly. Because numerous drug-resistant *P. falciparum* have been reported since 1961, current chemotherapy of malaria must focus on the increasing prevalence of drug-resistant *falciparum* malaria. In an authoritative review on the current status of malarial chemotherapy by Sweeney (2), the author points out that over the last 20 years, the brunt of the effort to develop new drugs against chloroquine-resistant malaria was borne by the U.S. Army. Of the more than 250,000 agents screened by the U.S. Army a number of potentially useful compounds have emerged from this program. The more promising of these have been described in an excellent review by Rozman and Canfield (3). The most promising of these is mefloquine hydrochloride, a blood schizonticide of the quinolinemethanol class of antimalarials.

## 2. Description

### 2.1 Nomenclature

2.11 Systematic Name

(±) Erythro-α-(2-piperidyl)-2,8-bis(trifluoromethyl)-4-quinolinemethanol hydrochloride

2.12 Common Name

Mefloquine hydrochloride<sup>a</sup>

2.13 Chemical Abstracts Registry Numbers

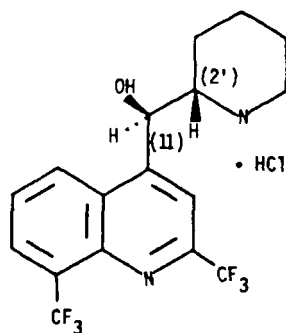
51773-92-3

2.14 Walter Reed Number

WR-142490

2.2 Formula2.21 Empirical

$C_{17}H_{16}F_6N_2O \cdot HCl$

2.22 Structural2.23 Absolute Stereochemistry

Erythro racemate (-)11S,2'R and (+)11R,2'S

<sup>a</sup>Although the mefloquine free base has been conjugated with acids other than hydrochloric, in this text mefloquine will mean mefloquine•HCl for the sake of brevity.

## 2.24 Molecular Weight

414.8

## 2.3 Appearance, Color, and Odor

Mefloquine is an odorless, white powder.

## 3. Physico-chemical Properties

### 3.1 Elemental Analysis

The elemental analysis of a typical bulk sample of mefloquine is as follows:

<u>Element</u>	<u>% Calculated</u>	<u>% Found</u>
C	49.23	49.42
H	4.13	4.15
Cl	8.55	8.63
F	27.48	27.29
N	6.75	6.96

### 3.2 Melting Characteristics

Employing the USP method and inserting the capillary at 245°C, mefloquine melts with decomposition 253–255°C, corrected.

### 3.3 Spectral Characteristics

#### 3.31 Infrared

Recorded as a Nujol mull on a Perkin-Elmer model 599B spectrometer, the spectrum, Figure 1, shows an abundance of fine structures, offering an excellent mean of identifying mefloquine. The readily assignable absorption bands are listed below.

<u>Wavenumbers cm<sup>-1</sup></u>	<u>Assignment</u>
3240	OH
3100–2500	R <sub>2</sub> NH <sub>2</sub> <sup>+</sup>
1600, 1585, 1515	aryl
1305, 1140	CF <sub>3</sub>

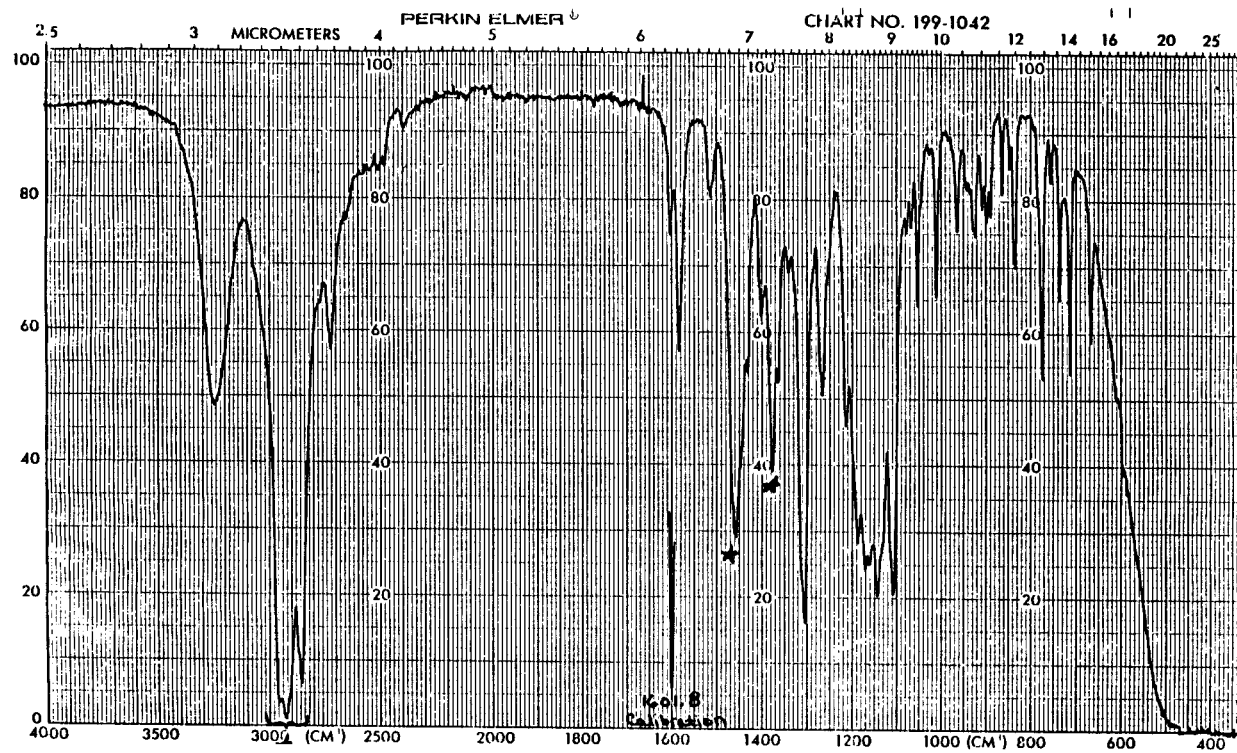


Figure 1



### 3.32 Ultraviolet

Recorded as solutions in N/10 HCl on a Cary Model 15 spectrometer, the spectra, Figure 2, show  $\lambda_{\max}$  near 317, 303, 283, and 222 nm. The calculated absorptivities are listed below:

317nm =	3,400
303nm =	4,430
283nm =	5,740
222nm =	41,800

### 3.33 NMR

#### 3.331 $^1\text{H}$

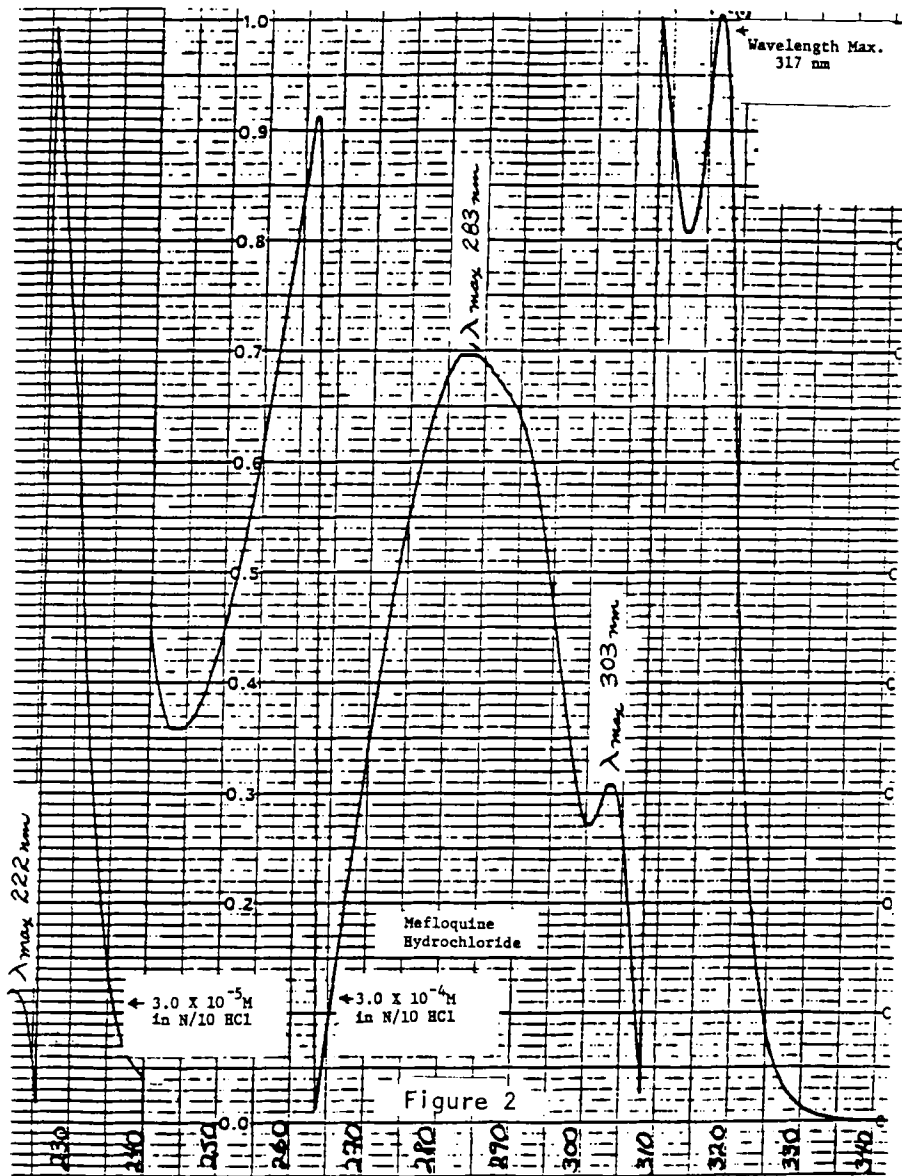
Recorded as a solution in DMSO- $d_6$  on a JEOL FX90Q, the spectrum, Figure 3, agrees well with the structural formula. Assignments of the various proton resonances are listed below.

Proton(s) at carbon	Chemical shift (ppm)	Resonance pattern
C-3	8.013	s
C-5	8.273	d
C-6	7.883	t
C-7	8.928	d
C-11	6.038	d, unresolved
C-11,OH	6.714	d
C-2'	~2.90	m
C-3'	~1.60	m
C-4'	~1.20	m
C-5'	~1.60	m
C-5'	~3.27	m

The C-11 protons in the threo racemate resonate at a higher field and show a larger coupling constant, ~5.8 ppm, ~7 Hz, than the corresponding protons in the erythro racemate, ~6.0 ppm, ~2 Hz (4).

#### 3.332 $^{13}\text{C}$

Recorded as a solution in DMSO- $d_6$  on a Varian XL-100, the spectrum, Figure 4, is in agreement with the indicated structure. Tentative assignments to the individual resonances are listed below.



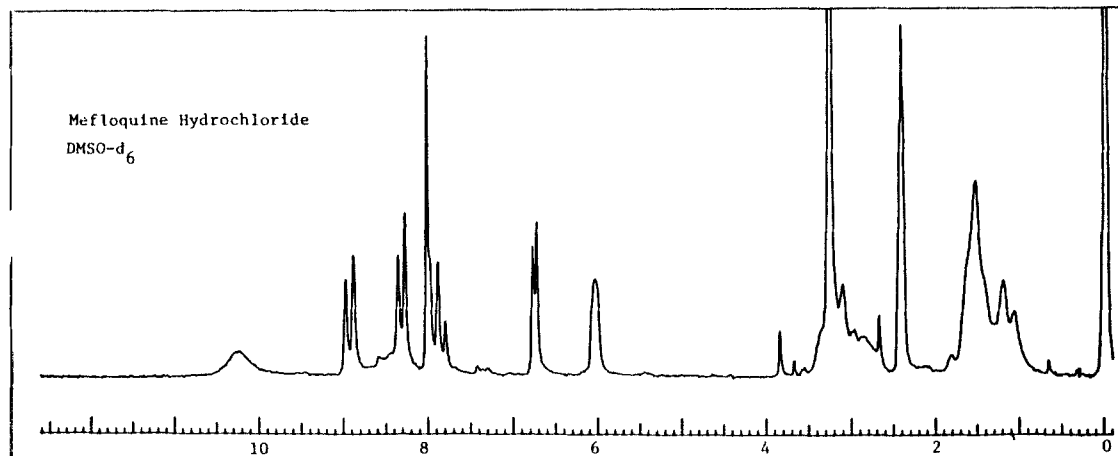
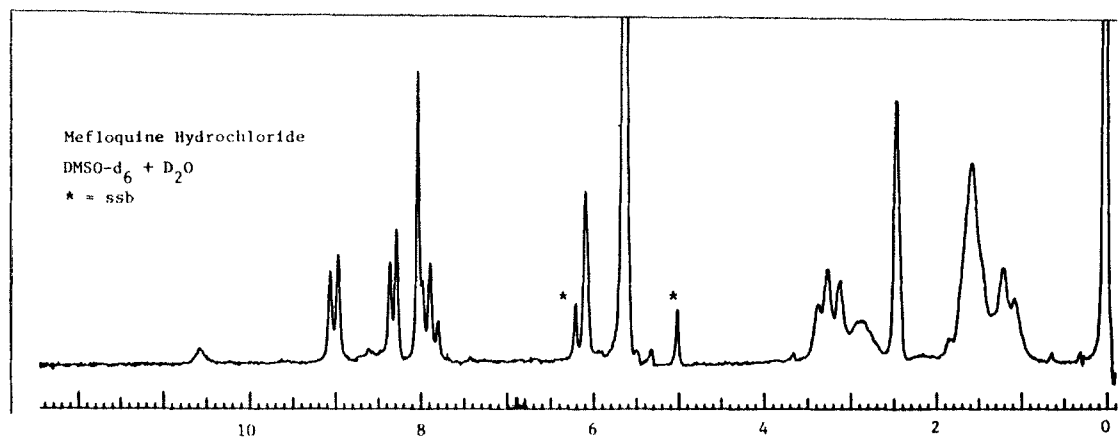


Figure 3

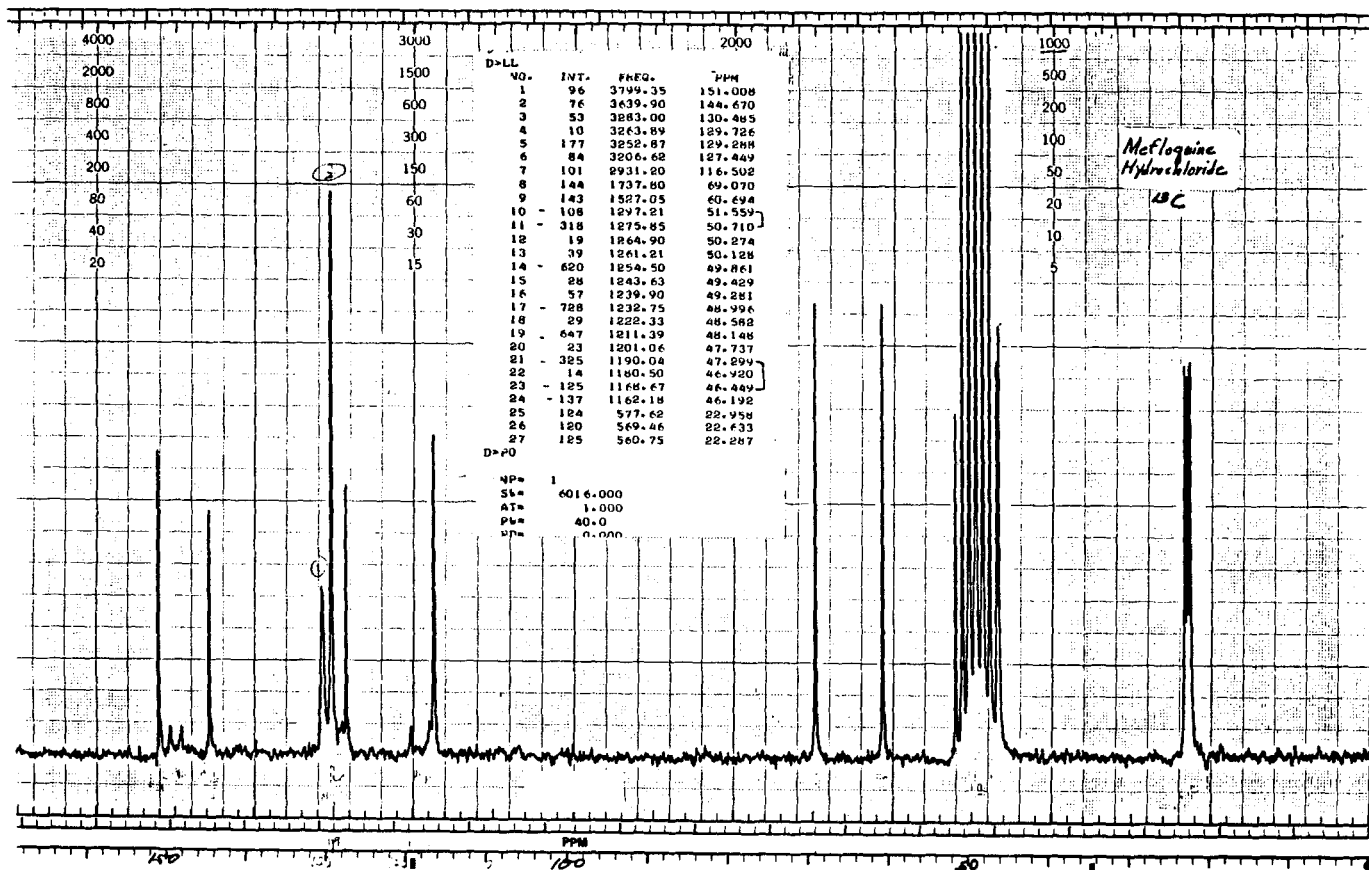


Figure 4

<u>Carbon</u>	<u>PPM</u>
2	~148.5 (q)
3	116.502
4	151.008
5 }	130.485
6 }	129.228
7 }	
8	~118.4 (q)
9	127.449
10	144.670
11	69.070
2'	60.070
3' }	{ 22.633
5' }	{ 22.958
4'	22.287
6'	46.192

### 3.333 <sup>19</sup>F

Recorded as a solution in DMSO-d<sub>6</sub> on a JEOL FX90Q and using sodium trifluoroacetate as the internal reference standard, the spectrum, Figure 5, is in agreement with the depicted structure. The C-2 and C-8 trifluoromethyls resonate at 16.707 and 8.817 ppm, respectively.

## 3.34 Mass Spectrometry

### 3.341 Chemical Ionization (CI)

A CI spectrum, Figure 6, determined on a Reibermag R-10 spectrometer and using ammonia as the reagent gas, shows the molecular ion at m/z 378.

### 3.342 Electron Impact (EI)

An EI spectrum, Figure 7, determined on a LKB 9000 spectrometer, shows a m/z 377 (M-H)<sup>+</sup>, m/z 359 (M-F)<sup>+</sup>, and m/z 84 (piperidyl)<sup>+</sup>, the base peak.

## 3.4 Solubility

### 3.41 Approximate Room-Temperature Solubilities

Solubilities of mefloquine were determined in several solvents. Results reported are based on the U.S.P. definitions (5).



TITLE LIM WR-142490 HCL-AS NOCI-NH3  
 0657 DA SCAN NO: 01 EKGD: X100% R.T. = 00:00:16 100% = 978944 SIGMA= 73%  
 DATE: 04/26/1984

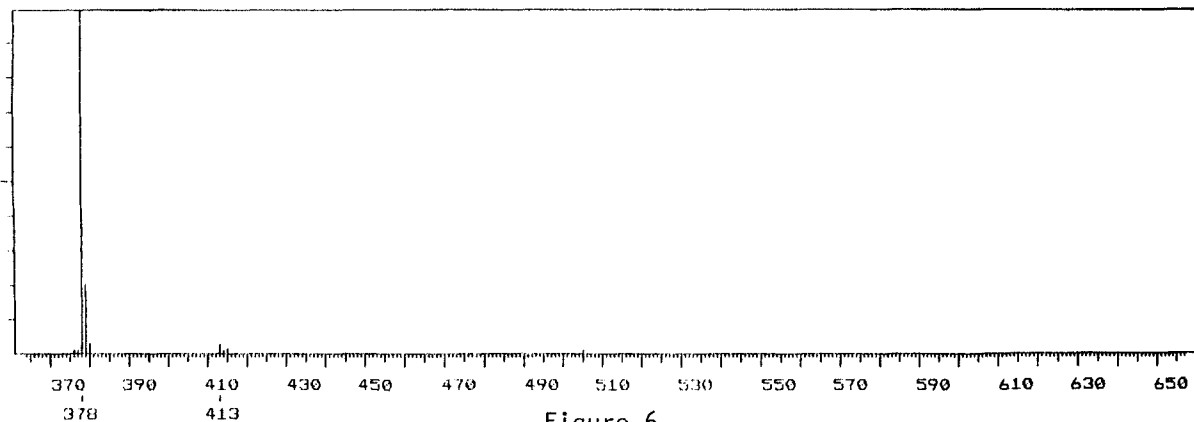
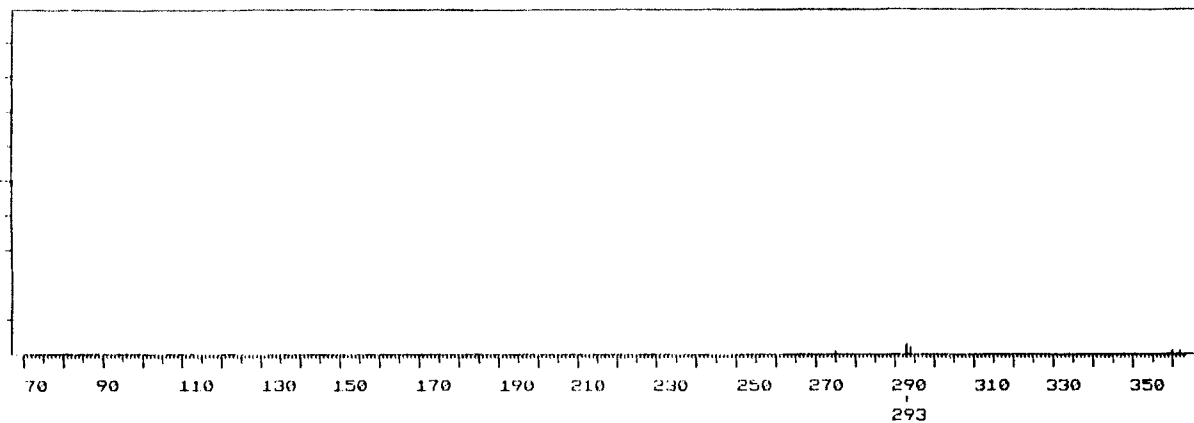


Figure 6

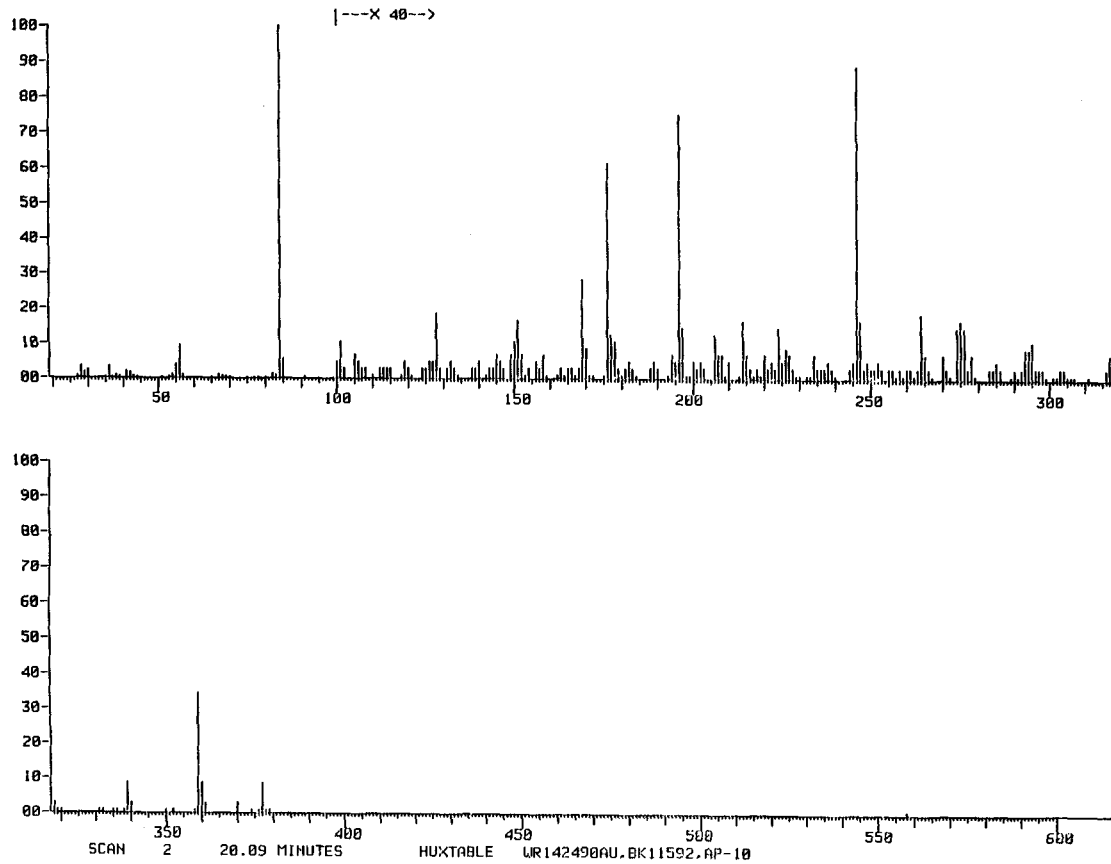


Figure 7



<u>Solvent</u>	<u>Solubility</u>
Water	slightly soluble
Dimethylsulfoxide	sparingly soluble
Methylene chloride	sparingly soluble
Ethyl acetate	soluble
Ethanol	soluble
Hydrochloric acid, 0.1M	slightly soluble

### 3.42 Partition Coefficient ( $K_p$ )<sup>b</sup>

<u>Organic Phase</u>	<u><math>K_p</math></u>
Benzene	34
n-Butanol	1300
Chloroform	0.1
Ether	94
Ethyl acetate	48
n-Heptane	2
Hexane	3

### 3.5 Ionization Constant ( $pK_a$ )<sup>c</sup>

Because mefloquine is only slightly soluble in aqueous media,  $pK_a$  determinations were carried out in aqueous ethanol. The experimentally found  $pK_a$  values are 8.54, 8.50, and 8.42 in 30, 50, and 70% aqueous ethanol; the extrapolated value for water is 8.6.

## 4. Synthesis

### 4.1 Overall Preparation

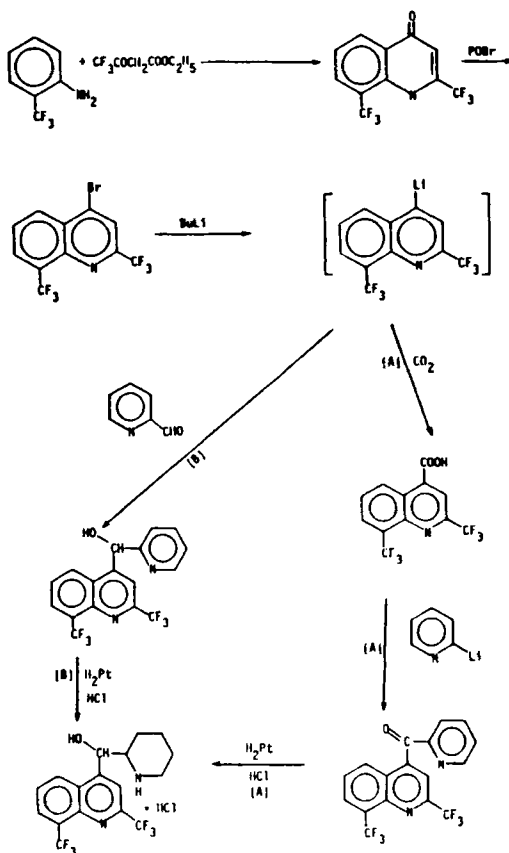
The preparation of mefloquine was first reported by Ohnmacht et al. (6). The procedure is depicted in scheme 1, pathway [A]. Although the catalytic reduction of the 2-piperidyl ketone results in two chiral centers, only the erythro racemate was reported. When mefloquine was prepared on a large scale by Olsen (7), the other racemate, the threo, was also found. An improved method of synthesis, patented by

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<sup>b</sup> $K_p$  is expressed as the ratio of concentration in the upper phase to that in the lower phase; these values are taken from Mu et al. (16).

<sup>c</sup>Taken from Mu et al. (16).

Grethe and Mitt (8) involved treating the lithio derivative of 4-bromo-2,8-bis(trifluoromethyl)quinoline with 2-pyridinecarboxaldehyde followed by hydrogenation of the resulting  $\alpha$ -(2-pyridyl)-2,8-bis(trifluoromethyl)-4-quinolinemethanol. This procedure is depicted in Scheme 1, pathway [B]. The patent claims only the erythro racemate was isolated.



Scheme 1

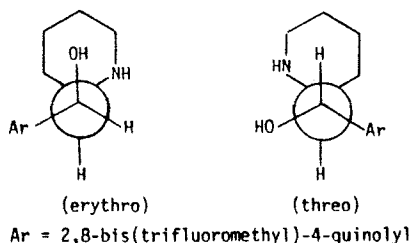
#### 4.2 <sup>14</sup>C-labelled Preparation

Both erythro (mefloquine) and threo racemates of  $\alpha$ -(2-piperidyl)-2,8-bis(trifluoromethyl)-4-quinolinemethanol-<sup>14</sup>C hydrochloride have been prepared by Yanko and Deebel (9), using the method of Ohnmacht et. al (6). Carbonation of the lithiated quinoline derivative with <sup>14</sup>carbon dioxide produced the 2,8-bis(trifluoromethyl)cinchoninic acid. Following

Scheme 1, pathway [A] produced the two racemates with the methanol carbon labelled in an erythro to threo ratio of 5.6.

#### 4.3 Optical Isomer Preparation

Carroll and Blackwell (4) prepared all four of the



optical isomers. Mefloquine, the erythro racemate, as the hydrochloride, was resolved via the formation of salts with (+)-3-bromo-8-camphorsulfonic acid. The preparation of the (+)- and (-)-threo enantiomers was carried out from both the (+)- and (-)-erythro enantiomers via the N,O-diacetyl derivative, selective hydrolysis of the latter to the N-acetyl derivative, inversion of the N-acetyl derivative to the O-acetyl derivative (presumably through oxazoline formation) and, finally, hydrolytic deacetylation. That the relative configuration of the erythro and threo racemates were correctly assigned was confirmed by acyl migration and NMR studies. The absolute stereochemistry, tentatively assigned by circular dichroism studies of the four optical isomers, indicates that the (-)- and (+)-erythro enantiomers have the 1S, 2R, and 1R, 2S configurations and the (-)- and (+)-threo enantiomers the 1S, 2S and 1R, 2R, respectively.

### 5. Analytical Methods

#### 5.1 Qualitative

##### 5.11 Spectral

Mefloquine is most readily identified by its spectral characteristics. Its infrared spectrum (Sect. 3.31) shows an abundance of fine structures and offers an excellent mean of overall identity. In our study on solid phase infrared spectra of numerous bulk samples and mefloquine isolated from dosage forms under a variety of conditions, no polymorphism has been observed.

Its proton spectrum easily distinguishes the erythro racemate from the threo and delineates the substitution pattern on the quinoline system (Sect. 3.331). Its EI mass spectrum establishes a molecular weight and a characteristic piperidyl ion (Sect. 3.342). Additional evidence of identity can be found in its  $^{13}\text{C}$  and  $^{19}\text{F}$  nmr spectra (Sect. 3.332, 3.333). Its UV spectral data, although less definitive than the aforementioned spectral characteristics, are nevertheless supportive of the quinoline system.

## 5.12 Chromatography

### 5.121 Thin-Layer

Thin-layer chromatographic profiles of bulk mefloquine are readily obtained by applying the material on silica-GF and developing with toluene:ethanol:conc  $\text{NH}_4\text{OH}$  (34:15:1)<sup>d</sup>. When detected by UV (254 and 365nm) and iodine vapor, the profile shows  $\text{NH}_4\text{Cl}$  at or very near the origin, mefloquine near  $R_f$  0.35, and, if present, the threo racemate near  $R_f$  0.50.

When developed in 2-propanol:conc  $\text{NH}_4\text{OH}$  (9:1),  $\text{NH}_4\text{Cl}$  remains at or near the origin, mefloquine near  $R_f$  0.55, and the threo racemate near  $R_f$  0.65.

An acidic mobile phase, hexane: $\text{HOAc}$ : $n$ -BuOH (8:1:1) has also been employed. This system, requiring multiple developments, is less practical and more difficult to reproduce. After ten developments, mefloquine moves to near  $R_f$  0.40 and, when present, the threo racemate to  $R_f$  0.20.

### 5.122 Gas

Being a conjugate acid of a dibasic amine, mefloquine has limited vapor pressure. To enhance its volatility, mefloquine is usually trimethylsilylated prior to being gas chromatographed. In our hands, we have not been able to obtain a satisfactory gc profile of mefloquine because of multiple species. Incomplete derivatization results in a mixture of the O-TMS derivative and underivatized mefloquine. Prolonged derivatization yields a small amount of a silylated carbamate, in addition to the expected O-TMS and

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<sup>d</sup>All solvent ratios are on volume:volume basis.

the N,O-diTMS derivatives. The carbamate is the piperidyl-CO<sub>2</sub> addition product; silylated along with the carbinol, the resulting derivative is the O,NCOO-diTMS. The O,NCOO-diTMS derivative increases concomitantly with the N,O-diTMS derivative, both increases at the expense of the O-TMS derivative. Silylation of the mefloquine free base under comparable conditions yielded a larger amount of the O,NCOO-diTMS derivative. Nakagawa et al. (10) employed the O-TMS derivative in a gc procedure to measure mefloquine in whole blood, but did not report the formation of other mefloquine-TMS derivatives.

### 5.123 High-performance Liquid

Grindel et al. (11) reported the uses of  $\mu$ Bondapak CN and  $\mu$ Bondapak C<sub>18</sub> columns to separate mefloquine from coextracted components found in biological fluids. In our laboratory, we have found a silica column and MeOH:hexane: conc NH<sub>4</sub>OH (80:19:1) to be a useful qualitative system. At a flow rate of 1.5 ml per min, mefloquine elutes near 6 min and the threo racemate near 4 min. In some of the bulk samples we have analyzed, one or two other trace impurities, faster eluting than mefloquine, are found. Because of the deleterious effect of NH<sub>4</sub>OH on silica, we have also employed a Hamilton PRP-1 column with THF:0.8% aq. conc NH<sub>4</sub>OH (48:52) at 0.8 ml per min and a  $\mu$ Bondapak CN column with 0.05M KH<sub>2</sub>PO<sub>4</sub> containing 0.1% HOAc:ACN:THF (67:24:9) at 2.0 ml per min.<sup>4</sup> The latter two systems do not separate the threo racemate from mefloquine nearly as well as the first.

## 5.2 Quantitative

### 5.21 Bulk Chemical

The most specific methods of assay are those based on a chromatographic separation followed by an optical measurement of the analyte. When possible, we employed the internal standard (IS) method for quantitation. We have used, as IS, 2-8-bis(trifluoromethyl)-4-[1-(hydroxy-3-N-t-butylamino)-propyl]quinoline phosphate, WR-184806, on a PRP-1 column with THF:0.8% aq. conc NH<sub>4</sub>OH (48:52) at 0.8 ml/min, and monitored at 285nm. We also have utilized m-nitrophenol as the IS on a  $\mu$ Bondapak CN column with 0.05M KH<sub>2</sub>PO<sub>4</sub> containing 1% HOAc:ACN:THF (67:24:9) at 2.0 ml/min and monitored at 282nm. The two racemates are generally more readily separated by normal phase HPLC (Section 6:123) or by TLC (Section 6.121). Should the HPLC systems described just above fail to separate the racemates, the amount of the threo racemate in a mefloquine sample will need to be determined independently by TLC or

HPLC on  $\text{SiO}_2$ . The threo racemate, which is generally present at  $\leq 1.0\%$ , if at all, is then subtracted from the mefloquine result derived from the above quantitative LC procedures.

Assays of lower specificity include those based on spectrometry and titrimetry. The reliability of the results from these assay methods depend heavily on the sample chromatographic profile; the closer is the profile to homogeneity, the more reliable will be the assay results. UV spectrometry has been used; the absorptivities cited Section 3.32 are reliable within 1%. Titrimetry has also been employed, using acetous perchloric acid as the titrant, preceded by treatment of the sample solution with mercuric acetate.

## 5.22 Dosage Formulation

### 5.221 Content Uniformity

Mefloquine is available as 250 mg tablets. The active component can be identified by infrared spectrometry, generally as a Nujol mull, after isolation from the dosage form by extraction with abs. ethanol and removal of the solvent. If additional evidence for verification is needed, other spectral techniques described in Section 3.3 or 5.11 can be employed.

Assays of the individual tablets have been carried out by the LC procedures described in Section 6.21, after the mefloquine has been isolated from the finely ground tablets by extraction with abs. methanol.

### 5.222 Dissolution Test

The USP XX dissolution test, using simulated gastric fluid at  $37 \pm 0.5^\circ\text{C}$  and a basket rotating at  $100 \pm 5$  rpm, has been applied to mefloquine tablets. A typical tablet showed 49% of the active component was in solution after 30 min, 60% after 60 min, and 74% after 120 min.

### 5.223 Disintegration Test

The USP XX procedure for uncoated tablets has been applied to mefloquine tablets. A typical tablet disintegrated within 60 sec.

## 5.23 Determination in Biological Fluids

Grindel et al. (11) measured mefloquine in blood, plasma,

and urine by HPLC, using normal and reverse phase systems and the internal standard method of quantitation. Specimens were extracted with ethyl acetate and the extracts concentrated before being chromatographed. Whole blood and plasma extracts were chromatographed on a  $\mu$ Bondapak CN column, and urine extracts were chromatographed on a  $\mu$ Bondapak C<sub>18</sub> column. The sensitivity of the assays for mefloquine was 0.05  $\mu$ g/ml whole blood or plasma and 0.25  $\mu$ g/ml of urine using 5-ml samples.

Nakagawa et al. (10) also determined mefloquine in whole blood. These authors employed ion-pair or the usual solvent extraction, concentration of the extract, trimethylsilylation of the dried residue, and gas chromatography of the O-TMS-mefloquine. Quantitations were based on the internal standard method; the determination limit was 10 ng/ml whole blood when an electron capture detector was used.

Mendenhall et al. (12) have determined plasma mefloquine using a plastic ion-selective electrode. The method sensitivity for mefloquine is moderate (0.4  $\mu$ g/ml), but when the extracted mefloquine is alkylated with benzyl bromide, a 100-fold improvement in sensitivity over electrode detection of underivatized mefloquine is claimed.

Schwartz (13) reported a TLC method for determining mefloquine in whole blood or plasma. The analyte was extracted with isopropyl acetate, concentrated and applied. After development with dichloromethane:methanol:acetic acid (80:10:10) the plates were scanned at 300nm. Using the method, levels of mefloquine and its metabolite were followed in the plasma of subjects up to 3 months, following an oral dose of 1g of the drug.

## 6. Stability

### 6.1 Bulk

As a bulk chemical stored in a screw-capped, amber glass bottle, mefloquine has been found to be stable for at least 18 months at ambient temperatures (20-30°C), at least three months at 45°C, and at least two months at 60°C.

### 6.2 Solution

No solution stability data on mefloquine are available because of its poor solubilities in physiologically compat-

ible aqueous media and because its dosage formulation is a tablet.

### 6.3 Dosage Form

Mefloquine is formulated as a 250-mg tablet. When stored in a screw-capped amber glass bottle, the active component has been established to be stable for at least three months at room temperature (20–30°C), at least three months at 45°C, and at least two months at 60°C. However, the heated tablets showed substantial increases in disintegration times and definite decreases in dissolution rates.

## 7. Biopharmaceutics

### 7.1 Pharmacokinetics

The kinetics of mefloquine in 20 healthy male subjects after single oral doses of 250, 500, 1,000, and 1,500 mg have been studied by Desjardins et al. (14). Whole blood concentrations of the drug were measured periodically for 83 days after drug administration; the resulting concentration/time data were analyzed to determine the rates of absorption and elimination. Absorption from a tablet was slower and less complete than when the drug was administered as an aqueous suspension. The volume of distribution was quite large. The therapeutic efficacy and prolonged duration of prophylactic effect of a single dose of mefloquine was consistent with the finding of a mean whole blood half-life of 13.9 days.

Nakagawa et al. (10) reported on a time course of the whole blood mefloquine concentration in a beagle dog given a 250-mg tablet. These authors observed a rapid increase in blood mefloquine level up to the maximum at 3 hr, followed by a very slow decrease to a level of 18 ng/ml at 7 weeks.

Schwartz et al. (15) reported kinetics studies of mefloquine and one of its metabolites in the dog and in man. Human subjects were given 1 g mefloquine orally and absorption started rapidly within the first hr. Plasma levels rising then more slowly to reach a maximum of 0.9 to 1.0 µg/ml within 2 to 12 hrs. Thereafter, mefloquine showed a somewhat fluctuating decline to a level of approximately 0.5 µg/ml at 12 weeks.

The disposition of mefloquine-<sup>14</sup>C was studied in detail in the rat by Mu et al. (16). Despite its extensive binding to plasma proteins, high tissue/plasma concentration ratios



were found. Fecal excretion accounted for most of the drug and metabolites. In vitro studies with red cells indicated a high affinity of the drug for the cell membrane.

## 7.2 Metabolism

A study on the metabolism of mefloquine in rat has been reported by Jauch et al. (17). Mefloquine- $^{14}\text{C}$  was given intraperitoneally to rats and the metabolite patterns in feces, urine, bile and blood were compared.

The main component in the feces was mefloquine. In addition, 2,8-trifluoromethyl-4-carboxyquinoline, a hydroxylated derivative (hydroxyl on the piperidyl ring), 2,8-trifluoromethyl-4-hydroxymethylquinoline, and another metabolite in which the piperidine moiety was converted to a lactam were found. The 2,8-trifluoromethyl-4-carboxyquinoline was the main metabolite in the urine. The bile contained the parent, the acid, and the alcohol, partially as conjugates. The blood contained the parent and the acid as main components.

In an earlier study, Rozman et al. (18) orally administered mefloquine- $^{14}\text{C}$  to female mice and found a similar excretion pattern as when administered intraperitoneally, although there were noted differences.

## 7.3 Animal Toxicology (19)

The safety and toxicity of mefloquine has been established in multiple animal species. When given in doses greater than three times the equivalent human treatment dose for periods of time greater than one month, the drug occasionally causes nonspecific abnormalities of hepatic and renal function in laboratory animals. In a single rodent species given the equivalent of 2-3 times the usual human treatment dose each day for 3 months to 2 years, lesions were found ranging from reversible epididymal edema to epididymal fibrosis. Ocular lesions including retinal degeneration and fibrosis, and corneal and lens opacification were also observed. These lesions have not been observed at lower doses in rats or in other laboratory animals (mice, dogs or monkeys) given doses equivalent to those that produced the lesions in the rats.

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Frederick, Maryland. This is contribution No. 1732 to the Army Research Program on Malaria. The opinions expressed are those of the author and not necessarily those of the U.S. Army.

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Literature surveyed through May 1984.

# IOPANOIC ACID

Davide Pitrè

*Bracco Industria Chimica s.p.a.  
Milan, Italy*

1. Foreword, History, and Therapeutical Category	182
2. Description	182
2.1 Nomenclature	182
2.2 Chemical Names	182
2.3 Generic Names	182
2.4 Trade Names	182
2.5 Formula, Molecular Weight	183
2.6 Appearance, Color, Odor	183
3. Physical Properties	183
3.1 Spectra	183
3.2 Physical Properties of the Solid State	191
3.3 Solubility	194
4. Manufacturing Procedures	194
4.1 Synthesis	194
4.2 Separation of the Racemate	195
5. Stability	196
6. Analysis of Impurities	196
6.1 Free Iodine and Free Halides	196
7. Metabolism and Pharmacokinetics	196
7.1 Metabolism	196
7.2 Pharmacokinetics	197
7.3 Protein Binding	198
7.4 Acute Toxicity	198
8. Methods of Analysis	198
8.1 Elemental Analysis	198
8.2 Identification	198
8.3 Complexometric Analysis	199
8.4 Organically Bound Iodine	199
8.5 Chromatography	200
9. Determination of Iopanoic Acid in Body Fluid and Tissues	201
References	203

1. Foreword, History and Therapeutical category

Iopanoic acid is an orally administered cholecystographic agent which is rapidly absorbed by the gastroenteric tract and is excreted with bile.

The main application of the product is for diagnosis of bile stones, a very common disease (1,2).

Iopanoic acid was first studied in 1952 by Archer and Hoppe (3,4) and is the dominant medium for colecystography up to-day. The success of this X-Ray contrast medium has been remarkable if we consider that more than 40 million doses have been administered (5). Nowadays the visualization of bile stones is carried out by ultrasound too and therefore oral colecystography is less common.

2. Description

2.1 Nomenclature

2.2 Chemical Names

Benzenepropanoic acid, 3-amino- $\alpha$ -ethyl-2,4,6-triiodo

Hydrocinnamic acid, 3-amino- $\alpha$ -ethyl-2,4,6-triiodo

3-amino- $\alpha$ -ethyl-2,4,6-triiodohydrocinnamic acid

CAS Reg. N. [ 96.83-3 ]

2.3 Generic Names

Acidum Iopanoicum

Iopanoic Acid (USAN, INN)

Natrii iopanoas (NFN)

2.4 Trade Names

Bilopaco (Rovi, Madrid)

Cistobil (Bracco, Milano)

Coligraf (Estedo, Barcelona)

Holevid (Krka, Novo Mesto)

Neocontrast (Bama, Barcelona)

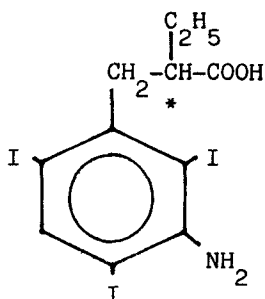
Nigrantil (Vinas, Barcelona)

Polognost (Polfa, Warszawa)  
 Telepaque (Sterling/Winthrop/Ross)  
 Teletrast (Astra)

#### 2.4.1 Trade Names of Sodium Salt

Bilijodan-Natrium (Leo)  
 Panjopaque (Lundbeck)

#### 2.5 Formula, Molecular Weight



$C_{11}H_{12}I_3NO_2$

Mol.Wt. 590.93

The asterisk indicates chiral center

#### 2.6 Apparence, color, odor

White to creamy-white powder, tasteless or nearly so; faint characteristic odor; is affected by light. (6,7,8,9,10).

### 3. Physical Properties

#### 3.1 Spectra

##### 3.1.1 Infrared Spectrum

The Infrared Spectrum of Iopanoic Acid is shown in Fig. 1. The Spectrum was recorded with a solid sample disc composed of 1 mg of Iopanoic acid/300 mg KBr. The following bands ( $cm^{-1}$ ) were assigned (11) and are reported in table 1.

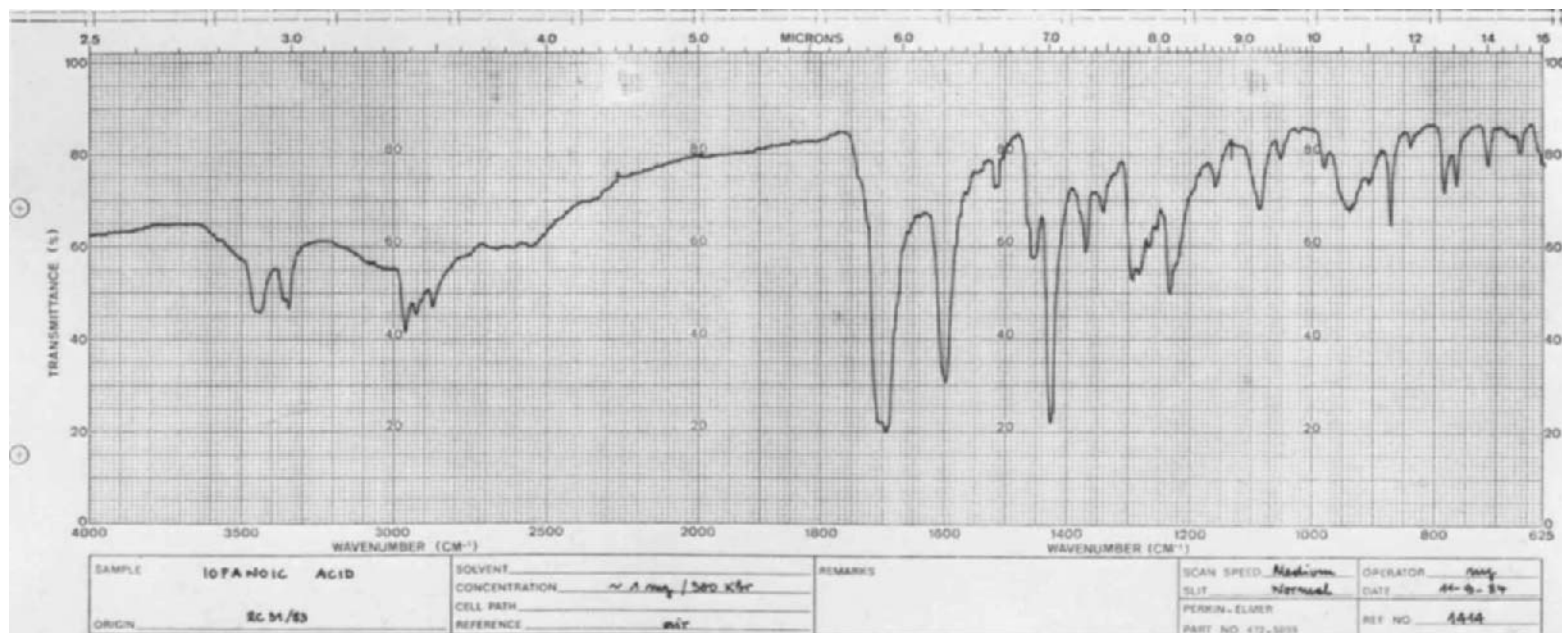


Fig. 1. Infrared Spectrum of Iopanoic Acid.

Table 1

<u>Wave number(<math>\text{cm}^{-1}</math>)</u>	<u>Assignment</u>
3435, 3445	$\sqrt{\text{NH}}$
1685	$\sqrt{\text{C=O}}$
1592	vibration of benzene ring
1453	$\delta\text{CH}$ of methyl and methylene
1422	$\sqrt{\text{C-O}}$ , $\delta\text{OH}$ dimer of carboxylic acid
1362, 1334	$\delta\text{CH}$ of methyl
1290	$\sqrt{\text{C-O}}$ of carboxylic acid
1229	$\sqrt{\text{C-N}}$
939	$\delta\text{OH}$ of carboxylic acid (out of plane)
872	$\delta\text{CH}$ of substituted benzene ring out of plane

### 3.1.2 Nuclear Magnetic Resonance Spectra

#### 3.1.2.1 $^1\text{H-NMR}$

$^1\text{H-NMR}$  Spectrum of Iopanoic acid, shown in Fig. 2, was obtained in  $\text{DMSO-d}_6$  with a Varian Spectrometer EM-390 operating at 90 MHz. The chemical shifts (12 ) are listed in the Table 2.

Table 2

<u><math>\delta\text{H}(\text{ppm, TMS})</math></u>	<u>Multiplicity</u>	<u>Number of protons</u>	<u>Assignment</u>
$\sim 12.6$	s, 1	1, exch.	$-\text{COOH}$
8.05	s	1	phenyl
5.28	s	2, exch.	$-\text{NH}_2$
3.21	d, d	2	$\phi\text{-CH}_2$
$2.8+2.4$	m	1	$-\text{CH}$
$1.95+1.1$	m	2	$\text{CH}-\text{CH}_2-\text{CH}_3$
0.83	t	3	$\text{CH}_2\text{CH}_3$



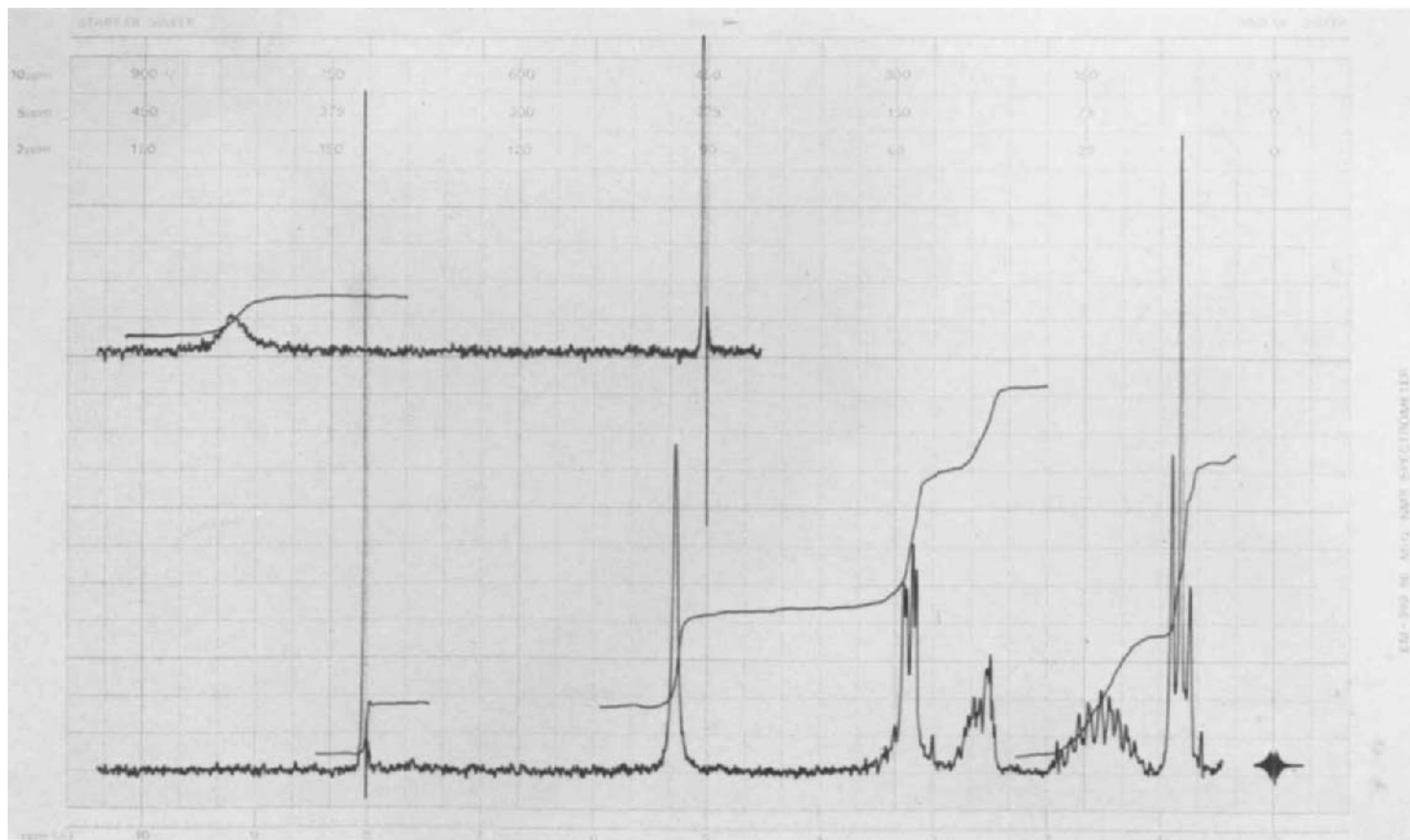


Fig. 2. H-NMR (90 MHz) Spectrum of Iopanoic Acid in DMSO-d<sub>6</sub>.

3.1.2.2  $^{13}\text{C}$ -NMR

The  $^{13}\text{C}$ -NMR Spectrum of Iopanoic Acid is shown in Fig. 3. The spectrum was recorded in  $\text{DMSO}-d_6$  with a Varian XL-100 Spectrum operating at 25.2 MHz. The chemical shifts (12) are listed in Table 3.

Table 3

<u>Line n.</u>	<u>Intensity</u>	<u><math>\delta\text{C}(\text{ppm}, \text{TMS})</math></u>	<u>Assignment</u>
1	250	175.0	C=O
2	327	147.6	aromatic carbon
3	160	146.7	"
4	291	143.8	"
5	216	88.4	"
6	201	83.95	"
7	167	80.2	"
8	139	47.3	-CH
9	224	46.3	$\phi\text{-CH}_2$
10	168	23.7	$\text{CH}_2\text{-CH}_3$
11	248	12.1	$\text{CH}_3$
12	97	0.0	TMS

3.1.3 Mass Spectrum

The Mass Spectrum of Iopanoic acid, shown in Fig. 4, was obtained by FAB technique with a VG-Micromass 70-70E instrument after mixing the sample with glycerine. The main and more characteristic fragmentation patterns are (13):

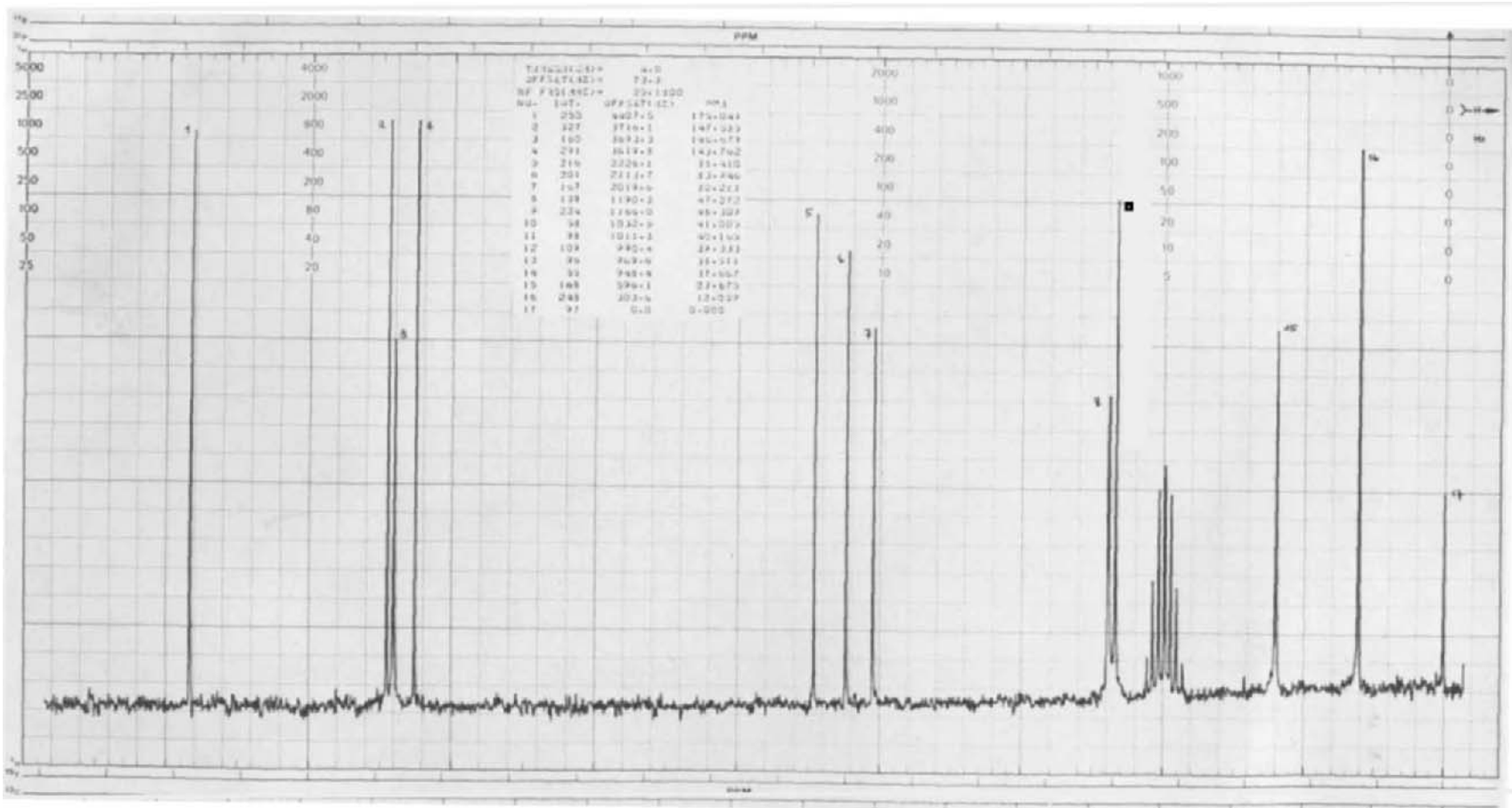


Fig. 3.  $^{13}\text{C}$ -NMR (25.2 MHz) Spectrum of Iopanoic Acid in  $\text{DMSO-d}_6$ .

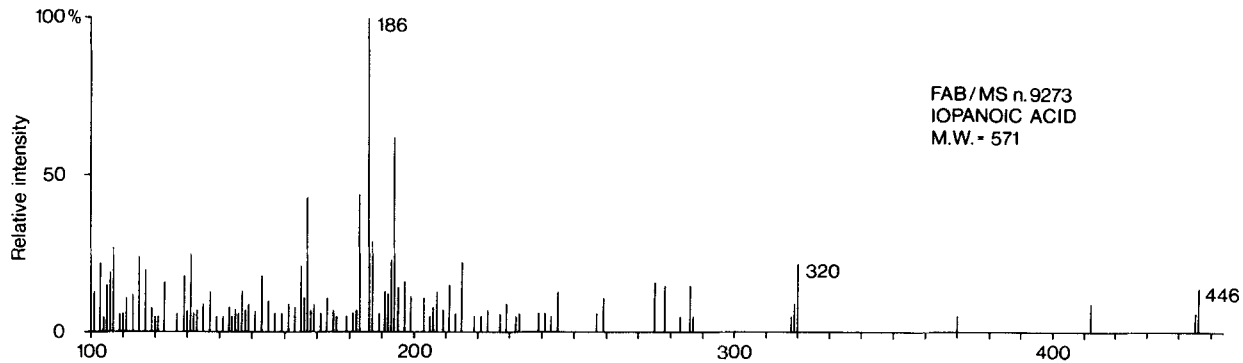
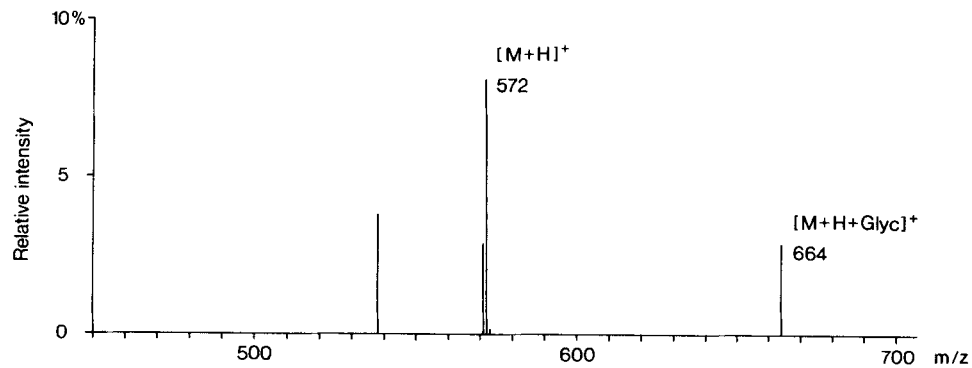
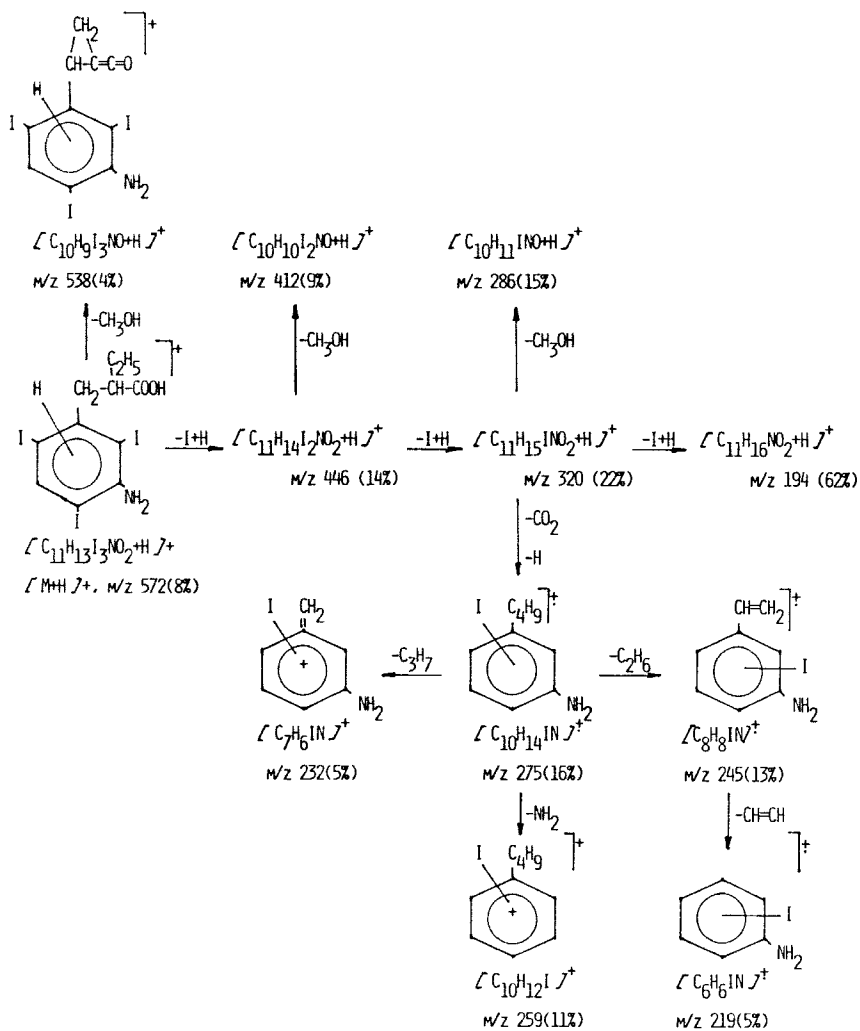


Fig. 4. FAB Mass Spectrum of Iopanoic Acid.



The complex ion  $[M+H+Glycerol]^+$ ,  $m/z$  664 and the sequences of deiodination and hydrogenation that give the  $m/z$  194 ion are common to other contrast media tested with the same technique.

#### 3.1.4 UV Spectra

The following extinction maxima and specific extinction coefficient of Iopanoic acid in ethanol were reported (14):

$$\lambda_{\max} = \text{nm } 231 \quad \lambda_{\max} = \text{nm } 315 \quad E_{1\%}^{1\text{cm}} = 629(231 \text{ nm})$$

### 3.2 Physical properties of the solid state

#### 3.2.1 Crystal Morphology

A study of polymorphism of Iopanoic acid (15) showed the existence of two polymorphous forms I and II. I is characteristic of the commercial product while II was obtained from a hot benzenic solution of I quickly frozen with a mixture of dry ice and acetone. There is also an amorphous form obtained by precipitation of Iopanoic acid from the aqueous solution of its sodium salt.

#### 3.2.2 Differential Thermal Analysis

The thermogram of form I shows a single endothermic peak at  $153.8^\circ$ , while that of form II has an endothermic peak at  $132.6^\circ$ , an exothermic peak at  $141.2^\circ$  followed by the melting peak at  $153.4^\circ$ . The thermogram of the amorphous form shows an endothermic transition peak at  $54.9^\circ$ , a broad exothermic peak at  $99.9^\circ$  followed by the melting peak at  $153.2^\circ$ (15).

#### 3.2.3 X-Ray Powder diffraction

The data were obtained with Phillips APD 3500 automated diffractometer using  $\text{CuK}\alpha$  ( $\lambda = 1.5413 \text{ \AA}$ ) monochromatic wavelength (15).

Table 4X-Ray Diffraction Patterns of Iopanoic Acid

Form I		Form II	
d, Å	I/I <sub>1</sub>	d, Å	I/I <sub>1</sub>
8.429	11.63	4.577	23.65
7.608	15.54	4.235	13.99
4.344	23.46	4.160	12.69
4.258	13.63	4.069	19.52
4.145	100.00	3.940	16.62
3.472	14.02	3.688	100.00
3.046	21.55	3.590	15.38
3.004	20.89	3.112	13.05
2.421	15.80	2.932	13.83
2.139	17.78	2.175	15.54

3.2.4 Melting Range

The following melting points are reported in different Pharmacopoeias for form I, i.e. the commercial product = M.p. 152°-8° (dec) (USP XX). About 155° (B.P. 1980). About 155° (Ph.Int.). M.p. 152°-8°(dec) (F.U.I. VII).

Thermal microscopy(15) shows that melting begins at 147° and is complete at 155°, which is in accordance with the values of 148°-55° found by other authors (16). The same technique shows for form II that melting begins at 131° and is complete at 135°.

3.2.5 Thermodynamic Data

Thermodynamic Data for three solid Iopanoic Acid Forms are reported in the following Table (15).

TABLE 5

## THERMODYNAMIC DATA FOR THREE SOLID IOPANOIC ACID FORM

FORM	ENTHALPIC TRANSITION TYPE								
	ENDOTHERMIC			EXOTHERMIC			MELT		
	TEMPERATURE	KCAL MOLE <sup>-1</sup>	$\Delta S, \text{EU}$	TEMPERATURE	KCAL MOLE <sup>-1</sup>	$\Delta S, \text{EU}$	TEMPERATURE	KCAL MOLE <sup>-1</sup>	$\Delta S, \text{EU}$
FORM I	--	--	--	--	--	--	153.8	6.62	15.5
							+0.2°	+0.09	+0.2
FORM II	132.6°	4.96	12.2	141.2°	-3.61	-8.71	153.4°	4.61	10.8
	+0.2°	+0.08	+0.2	+0.1°	+0.15	+0.15	+0.2°	+0.05	+0.1
AMORPHOUS	54.9°	0.662	2.02	99.9°	-3.60	-9.65	153.2°	6.85	16.1
	+0.1°	+0.079	+0.02	+0.5°	+0.09	+0.23	+0.3°	+0.23	+0.5



### 3.3 Solubility

Homogeneous solubility data (14) are listed in the following table.

Table 6

#### Solubility of Iopanoic acid

Solvents	Solub. mg/100 ml	1 mg/mg of solvent
H <sub>2</sub> O	1.5	66.600
CH <sub>3</sub> OH	3975	25
C <sub>2</sub> H <sub>5</sub> OH	3900	25
CHCl <sub>3</sub>	2291	40
(C <sub>2</sub> H <sub>5</sub> ) <sub>2</sub> O	6076	15

### 3.4 pKa

The ionization constant was determined by potentiometric titration in Methylcellosolve/water 80:20; extrapolation to aqueous solution gave a pKa = 5.06 (17). Other authors (18) reported a pKa = 5.9.

### 3.5 pH

After boiling and filtering a 1% aqueous suspension of Iopanoic acid, a pH = 5.45 (14) was measured.

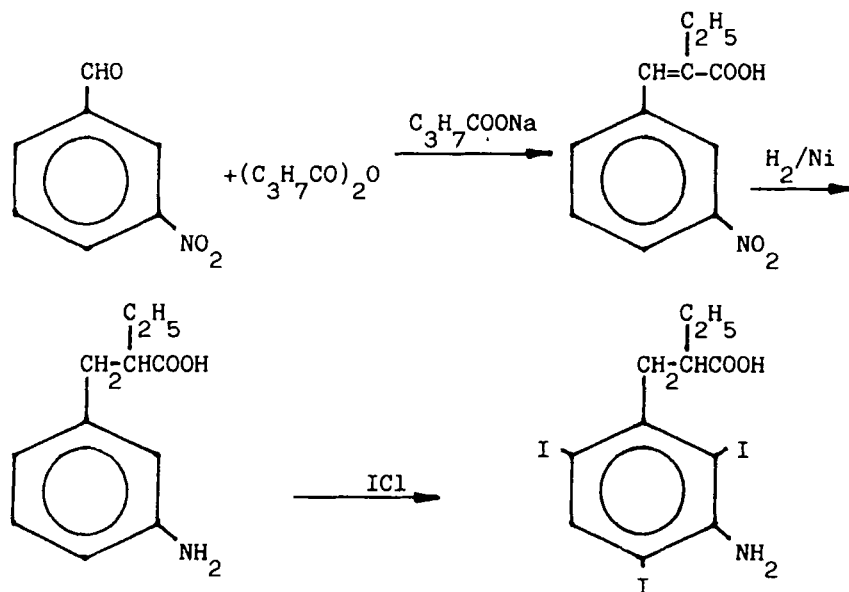
## 4. Manufacturing Procedures

### 4.1 Synthesis

Iopanoic acid may be synthesized in three steps (4) (19):

- a) Perkin condensation of 3-nitrobenzaldehyde with sodium butyrate and butyric anhydride to give m-nitro- $\alpha$  ethylcinnamic acid

- b) catalytic hydrogenation (Raney Nickel Catalyst) of m-nitro- $\alpha$ -ethylcinnamic acid to give 3-amino- $\alpha$ -ethylhydrocinnamic acid
- c) Iodination of the aminoacid with ICl in aqueous solution



<sup>125</sup>I labeled compound was obtained by iodine exchange with KI in acetamide at 140° for 6 hrs with a 14% radiochemical yield (20).

#### 4.2 Separation of the racemate

Optical resolution was carried out (21) according to Marckwald's technique (22) in EtOH solution with (-) and (+)  $\alpha$ -phenylethylamine. The absolute configuration of the two enantiomers was assigned (23)

R form m.p. 162°-3°  $[\alpha]_D^{20} = -5.2^\circ \pm 0.1^\circ$  (c=2 EtOH)

S form m.p. 162°  $[\alpha]_D^{20} = +5.1^\circ \pm 0.1^\circ$  (c = 2 EtOH)

5. Stability

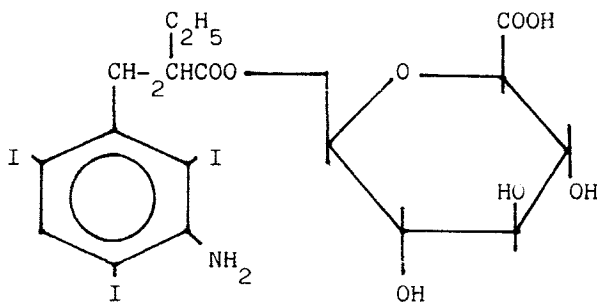
Iopanoic acid is chemically stable at room temperature but becomes slightly coloured when exposed to light (24).

6. Analysis of Impurities6.1 Free iodine and free halides

According to USP XX, absence of free iodine is detected by shaking 200 mg Iopanoic acid with 2 ml water and 2 ml chloroform. No violet colour must develop in the organic phase. BP 1980 tests the aqueous solution in which the acid has been reprecipitated and compares the colour of the chloroform layer with that of standard solution (20 ppm I). Halogen determination according to USP must give a turbidity lower than the one corresponding to 0.05 ml of 0.02N HCl.

7. Metabolism and Pharmacokinetics7.1 Metabolism

Metabolism studies following oral and parenteral administration of Iopanoic acid to cats (18,25) dogs (26) and humans (18) showed that the product is excreted in the bile as its glucuronide conjugate CAS Reg. N. / 58051-18-6/



The glucuronide was isolated and its structure confirmed (18). A method of isolation and purification of the metabolite from dog's bile has been reported (26).

Enterohepatic recirculation of the glucuronide was demonstrated in dogs (27). Recent metabolism studies (28) (29) of the two enantiomorphs of Iopanoic acid in rats evidenced a stereoselectivity in the excretion of glucuronides for the form corresponding to S(+) Iopanoic acid.

## 7.2 Pharmacokinetics

As regard human pharmacokinetics only data referring to blood levels (Table 7) and to urinary and fecal excretion, though not recent, are available (Table 8) (30).

Table 7  
Peak blood level in man

Dose g	Hours after dosage	Concentration mg/l	Note	Ref.
3	8-9	103-143	After 72h = 17 mg/	(31)
3	1-3	5-65	--	(32)
-	2	10-159	--	(32)
4	4-6	40-62	After 24h = 12 mg/l	(33)
2.7	4-6	65-124	After 24h = 12 mg/l	(34)

Table 8  
Excretion in man

Dose g	Hours after	Excretion % of dose		Plasma half life hrs	Ref.
		urinary	Fecal		
-	-	-	-	9	(34)
3	108	28-45	57-58	--	(18)
3	108	27-44	--	--	(35)
3	120	18	--	--	(32)
3	120	10-14	70-82	--	(36)

### 7.3 Protein Binding

HSA-binding curves suggest the presence of two or three independent binding sites (37,38) with 97.7% total binding to serum albumins. No significant differences in protein binding were observed using albumines from ox, rabbit, horse, pig, sheep and dog (37).

### 7.4 Acute toxicity

The acute toxicity ( $DL_{50}$ ) of Iopanoic acid was found to be:

in the mouse =  $320 \pm 20$  mg/kg ip (4) 285 mg/kg ip (39)

6.6 g/kg p.o. (solid) (1) 1.5 g/kg p.o.  
(solution) (39)

in the rat = 320 mg/kg ip and 2870 mg/kg po (solution)(39)

in the dog =  $300 < DL_{50} < 450$  mg/kg (39).

## 8. Methods of Analysis

### 8.1 Elemental Analysis

Element	% Theory
C	23.14
H	2.12
I	66.69
N	2.45
O	5.60

### 8.2 Identification

The following identification tests can be used according to J. Ph. IX.

- 1) A 0.1 g sample, heated on a flame, must evolve iodine vapours.
- 2) The IR Spectrum taken in KBr disk shows absorption maxima at 3435, 3345, 1290, 1229, 939, and  $872\text{ cm}^{-1}$ ,. B.P. 1980 reports the reference spectrum of Iopanoic acid.

- 3) 3 ml dilute HCl are added to a solution of 1 mg iodinated product in 1 ml acetone.

The resulting solution must give the aromatic amine reaction.

USP XX, Ph Int II and FUI VII test the iodide developed in aqueous solution after alkaline fusion with  $\text{Na}_2\text{CO}_3$ .

### 8.3 Complexometric Analysis

The method is based on the formation of an insoluble calcium salt in an aqueous solution of  $\text{CaCl}_2$ . The excess reagent is titrated with EDTA (Eriochrome Black T indicator) (40).

### 8.4 Organically bound iodine

All Pharmacopoeia methods consist in the reduction of bound iodine by means of Zn in alkaline solution: titration of  $\text{I}^-$  which follows is different.

Pharm.	Titrating solution	Indicator
USP XX	0.05 N $\text{AgNO}_3$	Tetrabromophenolphthalein ethyl ether
BP 1980	0.05 M $\text{KIO}_3$	Chromatic change
FUI VII	0.1N $\text{Na}_2\text{S}_2\text{O}_3$	Soluble starch

The method described by R. Wlodzinnerz (41) consists in a treatment with Zn or  $\text{KMnO}_4$ , acidification with  $\text{HNO}_3$  to pH 1-2, addition of an excess of standard solution of  $\text{AgNO}_3$  and titration of the excess with KI using  $\text{Cu}(\text{NO}_3)_2$  as indicator.

G.Y. Yakatan et al. (42) suggested for USP XVI and NF IX a standard procedure consisting in Oxygen flask combustion followed by absorption on alkaline sulphite and potentiometric titration with  $\text{AgNO}_3$  using an Ag-Calomel electrode pair.

Mineralization of Iopanoic acid to ionic iodine can be effected with good results using alkaline  $\text{KMnO}_4$  (43).

Mineralization may also be achieved in a microbomb of nickel using  $\text{Na}_2\text{O}_2$ ,  $\text{KNO}_3$  and sucrose.

The solution thus obtained is treated with  $\text{AgNO}_3$  and the isolated  $\text{AgI}$  dissolved in  $\text{KI}$  and determined as  $\text{Ag}$  by atomic absorption spectrophotometry at  $3280.7 \text{ \AA}$  (44). Organic iodine bound to the Iopanoic acid molecule is splitted off by treatment with  $\text{NaBH}_4$  in presence of  $\text{Pt}$ . After decomposition of sodium tetrahydroborate and acidification with  $\text{H}_2\text{SO}_4$ , iodide is titrated potentiometrically with  $\text{AgNO}_3$  using a silver/silver chloride electrode (45).

## 8.5 Chromatography

### 8.5.1 Paper Chromatography

A paper chromatographic assay has been reported (18) which uses the following system :

- Solvent system : butanol saturated with 2% aqueous boric acid;  $R_f = 0.83-0.86$
- Solvent system (46): Ethanol-25% ammonia 2:1;  $R_f = 0,80$
- Methods of detection: Ehrlich's reagent

### 8.5.2 Thin layer chromatography

The methods for separation and detection of Iopanoic acid are summarized in Table 9.

Table 9

<u>Solvent system</u>	<u>Plate</u>	<u>Rf</u>	<u>Reference</u>
I	A	0.64	(14)
II	B	-	(47)
III	B	0.82	(48)(26)
IV	C	0.74	(49)
V	C	0.15	(49)
VI	C	>0.95	(49)
VII	C	0.40	(49)
VIII	D	0.28	(20)
IX	D	0.33	(20)

- Solvent System

- I ethyl acetate:isopropanol:ammonia 25% (55:35:20)
- II butanol saturated with 2% boric acid
- III butanol saturated with 2% boric acid
- IV toluene:ethyl acetate:85% formic acid (50:45:5)
- V toluene:isopropanol:conc.ammonia (70:29:5)
- VI toluene:ethyl acetate:isopropanol:12 N acetic acid (10:35:35:20)
- VII toluene:dioxan:methanol:conc.ammonia (20:50:20:10)
- VIII acetone:acetic acid (99:1)
- IX Ethyl acetate:methanol:Acetic acid (80:20:1)

- Plate

- A = Kieselgel HF254
- B = Silica gel (Eastman A 13181)
- C = Kieselgel 60 F254
- D = Eastman polyethylene packed silica gel plate with fluorescent indicator

### 8.5.3 HPLC

A method for chromatographic separation by HPLC of Iopanoic acid and its glucuronide was described (26)(29). Separation is performed through an reverse phase C-18 column using a 1 ml/min flow and an isocratic phase acetonitrile-phosphate buffer 0.01M pH 4.3 (60/40, V/V). Iopanoic acid has a  $R_t \approx 11$  min.

### 9. Determination of Iopanoic acid in body fluids and tissues

The following methods are used for this determination:

- a) total iodine (18) according to the standard alkaline fusion procedure (50) followed by estimation of iodine.
- b) Total iodine determined with PBI Technicon autoanalyzer (51).



- c) Total iodine determination by fluorescent excitation analysis (FEA) (26).
- d) Total radioactivity of  $^{125}\text{I}$  material determined in a gamma counter (29). It is possible to quantify labeled Iopanoic acid and its glucuronide by means of HPLC.

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# LIDOCAINE BASE AND HYDROCHLORIDE

K. Gröningsson, J-E. Lindgren, E. Lundberg,  
R. Sandberg, and A. Wahlén

*Astra Läkemedel AB  
Södertälje, Sweden*

1. Foreword	209
2. Description	210
2.1 Structure	210
2.2 Name	210
2.3 Formula, Molecular Weight	210
2.4 Colour, Odour, Crystal Forms	210
3. Physical Properties	210
3.1 Melting Ranges	210
3.2 Solubility	213
3.3 Acid Dissociation Constant	213
3.4 Distribution Ratios	213
3.5 Infrared Spectra	213
3.6 Nuclear Magnetic Resonance Spectra	213
3.7 Ultraviolet Spectra	219
3.8 Mass Spectrum	222
4. Synthesis	222
5. Methods of Analysis	224
5.1 Elemental Analysis	224
5.2 Identification Tests	224
5.3 Titrimetry	225
5.4 Gas Chromatography	225
6. Stability—Degradation	226
7. Pharmacokinetics	227
7.1 Plasma Concentrations after Different Routes of Administration	227
7.2 Protein Binding	228
7.3 Placental Transfer	228
7.4 Biotransformation and Elimination	228
8. Methods of Analysis—Biochemical Applications	230
8.1 High Performance Liquid Chromatography (HPLC)	230
8.2 Gas Chromatography (GC)	230
8.3 Gas Chromatography—Mass Spectrometry, Selected Ion Monitoring	230
8.4 Homogeneous Enzyme Immunoassay, EMIT®	234
9. Identification and Determination in Pharmaceuticals	234
9.1 Identification Tests	234
9.2 Extraction Analysis	235
9.3 Gas Chromatography	235

9.4 Capillary Isotachophoresis	236
9.5 Liquid Chromatography	236
References	238

## 1. FOREWORD

In 1935 Swedish chemists engaged in the search for local anaesthetics at the institute for Organic Chemistry, University of Stockholm were investigating indole derivatives. Several organic compounds with local anaesthetic effect were synthesized. Further synthetic work by Löfgren, resulted in 1943 in the local anaesthetic compound lidocaine (1,2).

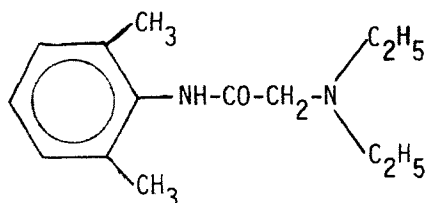
The clinical trials performed in co-operation with Astra, Sweden, showed that lidocaine had clear advantages over the available drug of choice, procaine. The documentation was presented to the Swedish regulatory authorities in 1947, including the trademark Xylocaine®.

Lidocaine was launched in Sweden in 1948 followed by France and Canada in the early fifties.



## 2. DESCRIPTION

### 2.1 STRUCTURE



### 2.2 NAME

1-Diethylamino-2',6'-acetoxylidide

### 2.3 FORMULA, MOLECULAR WEIGHT

Lidocaine base:  $C_{14}H_{22}N_2O$ ,  
molecular weight 234.34  
Lidocaine hydrochloride hydrate:  $C_{14}H_{22}N_2O \cdot HCl \cdot H_2O$ ,  
molecular weight 288.82

### 2.4 COLOUR, ODOUR, CRYSTAL FORMS

Both the base and the hydrochloride are white odourless substances. The base crystallizes from n-hexane as fine needles while the hydrochloride is obtained as a micro-crystalline powder from aqueous acetone

## 3. PHYSICAL PROPERTIES

### 3.1 MELTING RANGES

The measurements were performed in a Mettler FP-5 melting point apparatus (heating rate  $1^\circ\text{C}/\text{min}$ ) (4).

Lidocaine base	68 - $69.0^\circ\text{C}$
Lidocaine hydrochloride hydrate	76 - $79.0^\circ\text{C}$

In Figures 1 and 2 are shown the differential scanning calorimetry (DSC) thermal curves for the base and the hydrochloride respectively.  
The anhydrous hydrochloride melts at  $125^\circ\text{C}$ .

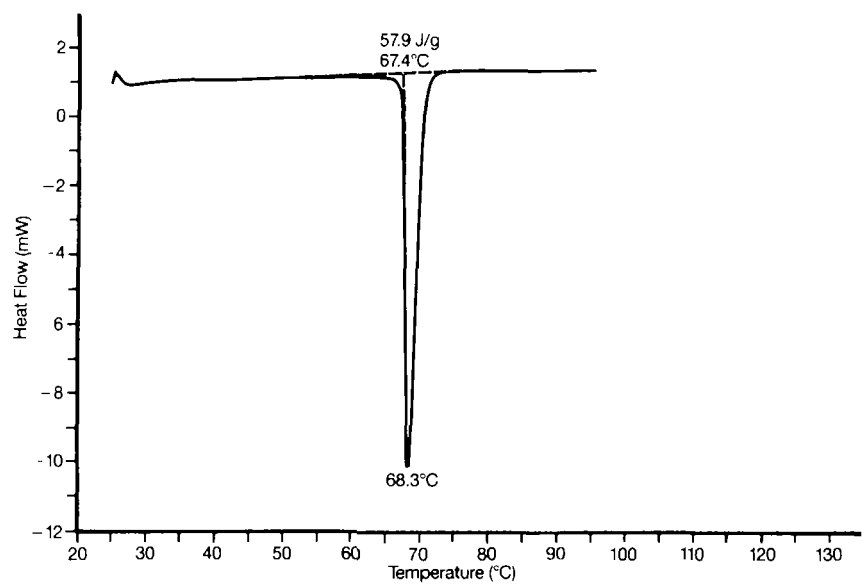


Fig. 1. DSC curve of lidocaine base

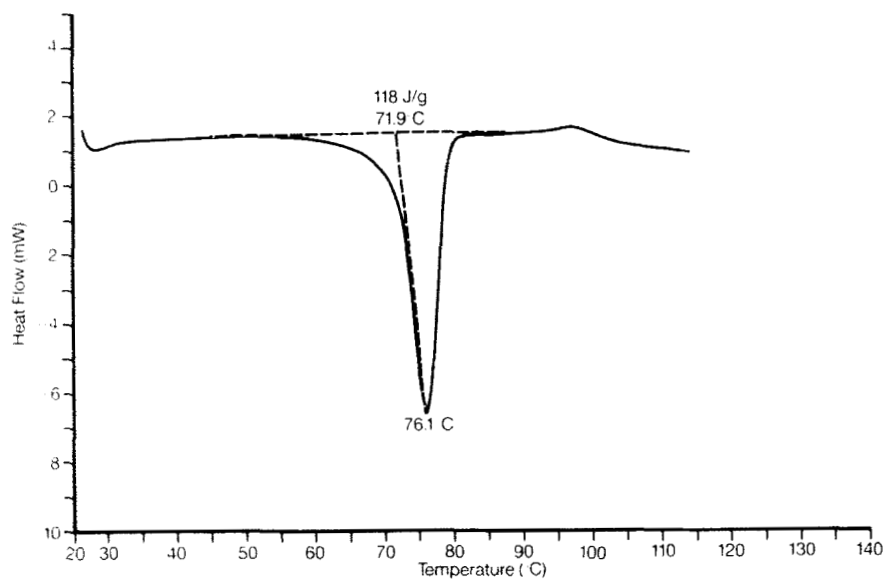


Fig. 2. DSC curve of lidocaine hydrochloride hydrate

### 3.2 SOLUBILITY

The solubility data given in Table 1 have been determined photometrically (4).

The temperature dependence for the solubility of the base in water is described by a  $\Delta H$  of  $-7.6 \pm 0.69$  (12) kJ/mol,  $\pm$  SE (df) (5). This dependence is in agreement with previously reported solubilities (6).

### 3.3 ACID DISSOCIATION CONSTANT

The acid dissociation constant  $pK'_{HA}$  defined by the equation  $K'_{HA} = a_{H^+} \times [A] \times [HA^+]^{-1}$  has been determined by potentiometric titration (3). At 25°C the value 7.84 was obtained

### 3.4 DISTRIBUTION RATIOS

The constants obtained are shown in Table 2.

### 3.5 INFRARED SPECTRA

The IR spectra are shown in Figures 3 and 4. They were taken as KBr discs with a Perkin-Elmer 298 spectrophotometer (4). The assignments are given in Table 3.

### 3.6 NUCLEAR MAGNETIC RESONANCE SPECTRA

The  $^1H$  NMR spectra are shown in Figures 5 and 6 and the  $^{13}C$  NMR spectrum (of the base) in Figure 7.

All the spectra were obtained on a Jeol FX 200 at 200 MHz (4). As solvents were used chloroform for the base with tetramethylsilane as internal standard and deuterium oxide for the hydrochloride with 1 % dioxan as internal standard (Tables 4 and 5).

Table 1. Solubility (g/ml, + 25°C) of lidocaine base and lidocaine hydrochloride hydrate

	Solvent			
	water	95% ethanol	chloroform	n-hexane
base	0.004	0.76	0.79	0.12
hydrochloride	0.68			

Table 2. Distribution ratios at + 25°C.

System	C <sub>org phase</sub> /C <sub>water phase</sub>	Ref.
n-octanol - phosphate buffer at pH 7.4	46	4
methylene chloride - water (base)	5200	3
toluene - water (base)	160	3

Table 3. Infrared Spectral Assignments for Lidocaine base and Lidocaine hydrochloride hydrate.

Lidocaine base		Lidocaine hydrochloride	
Band (cm <sup>-1</sup> )	Assignment	Band (cm <sup>-1</sup> )	Assignment
3240	amide N-H	3430, 3360, 3160	amide N-H
2960, 2790	C-H	2960	C-H
1660	amide I	2450	NH <sup>+</sup>
1590	C = C	1645	amide I
1490	amide II	1530	amide II
760	aromatic C-H	775	aromatic C-H

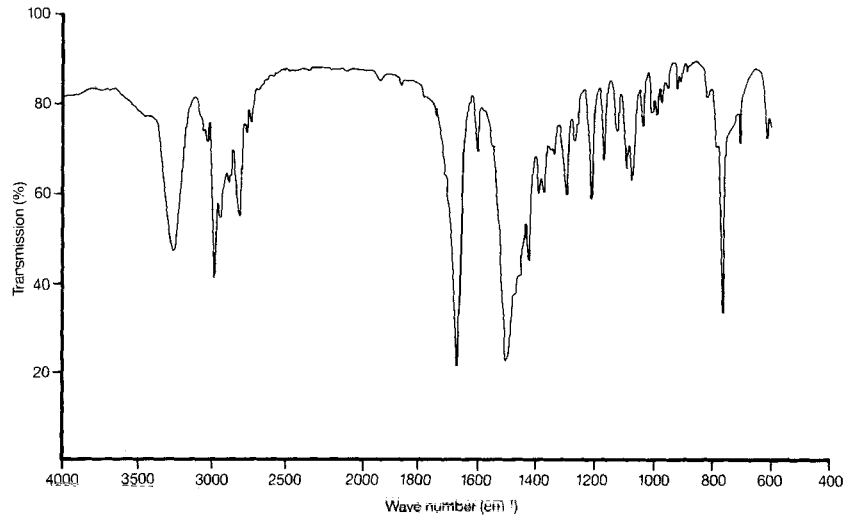


Fig. 3. IR spectrum of lidocaine base

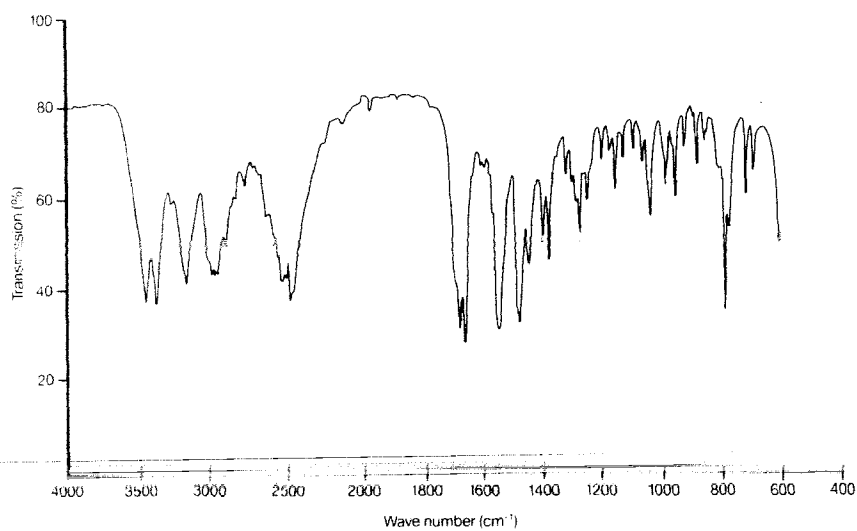


Fig. 4. IR spectrum of lidocaine hydrochloride hydrate

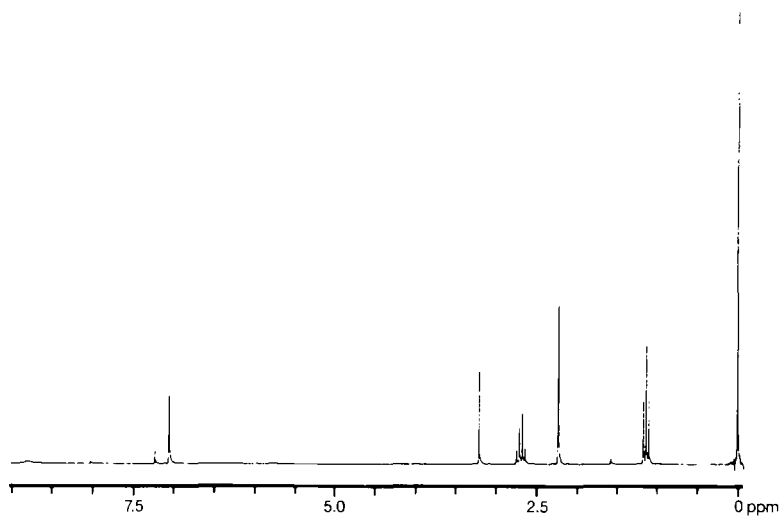


Fig. 5.  $^1\text{H}$  NMR spectrum of lidocaine base ( $\text{CDCl}_3$ )

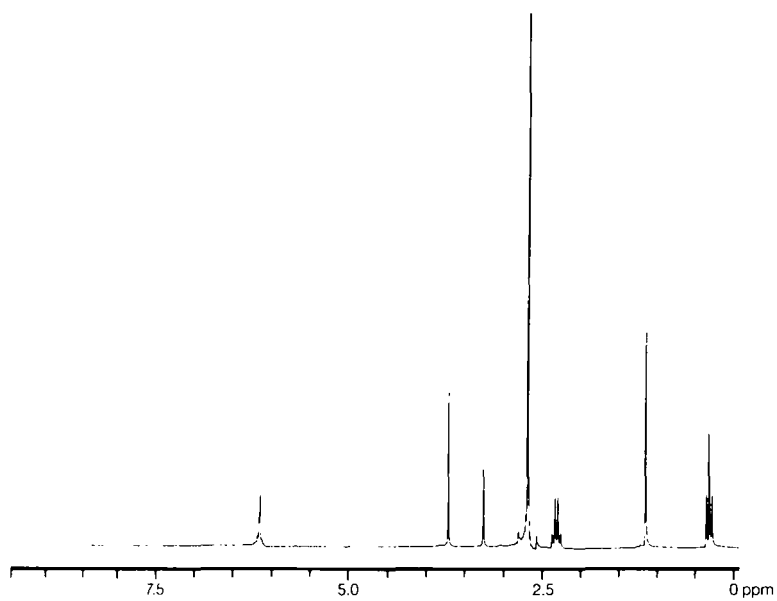


Fig. 6.  $^1\text{H}$  NMR spectrum of lidocaine hydrochloride hydrate ( $\text{D}_2\text{O}$ )



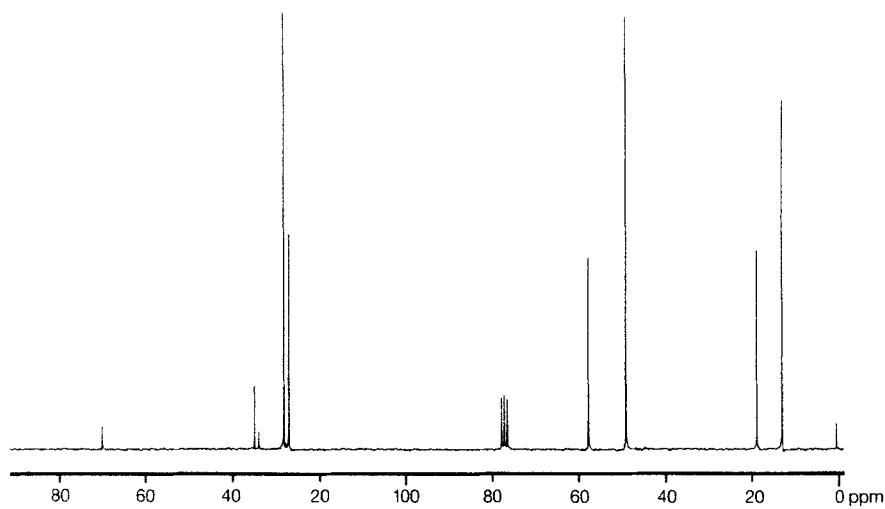


Fig. 7.  $^{13}\text{C}$  NMR spectrum of lidocaine base ( $\text{CDCl}_3$ )

Table 4.  $^1\text{H}$  NMR Assignments for Lidocaine base and Lidocaine hydrochloride hydrate

Group	$\text{CH}_2\text{CH}_3$	$\text{CH}_2\text{CH}_3$	$\varphi-\text{CH}_3$	$\begin{smallmatrix} \text{O} \\ \parallel \\ \text{CCH}_2\text{NH} \end{smallmatrix}$	Aromatic H
Chemical shift (ppm)					
base	1.14	2.69	2.23	3.21	7.07
hydrochloride	0.30	2.30	1.13	3.24	6.14
$^1\text{H}$ - $^1\text{H}$ spin coupling (J Hz)	t(7)	q(7)	s	s	s
base and hydrochloride					
Number of protons	6	4	6	2	3

Table 5.  $^{13}\text{C}$  NMR Assignments for Lidocaine base

Group	$\text{CH}_2\text{CH}_3$	$\text{CH}_2\text{CH}_3$	$\varphi-\text{CH}_3$	$\begin{smallmatrix} \text{O} \\ \parallel \\ \text{CCH}_2\text{NH} \end{smallmatrix}$	aromatic C unsubst.	aromatic C subst.	$\begin{smallmatrix} \text{O} \\ \parallel \\ \text{C} \end{smallmatrix}$
Chemical shift (ppm)							
base	12.6	49.0	18.5	57.7	127.0;128.2	134.1;135.1	170.1

### 3.7 ULTRAVIOLET SPECTRA

The UV spectra were taken with a Hewlett-Packard 8450 A spectrophotometer and are shown in Figures 8 - 11 (4). The data are summarized in Table 6.

Table 6. Data from UV-spectra, lidocaine base and lidocaine hydrochloride hydrate

	Solvent	$\lambda$ nm	$\epsilon$	Conc. mol/liter
Base	$\text{H}_2\text{O}$	262	420	$1.089 \cdot 10^{-3}$
Hydrochloride	"	262	455	$0.926 \cdot 10^{-3}$
Base	EtOH, 99.5%	262	348	$1.052 \cdot 10^{-3}$
Hydrochloride	"	263	377	$0.921 \cdot 10^{-3}$

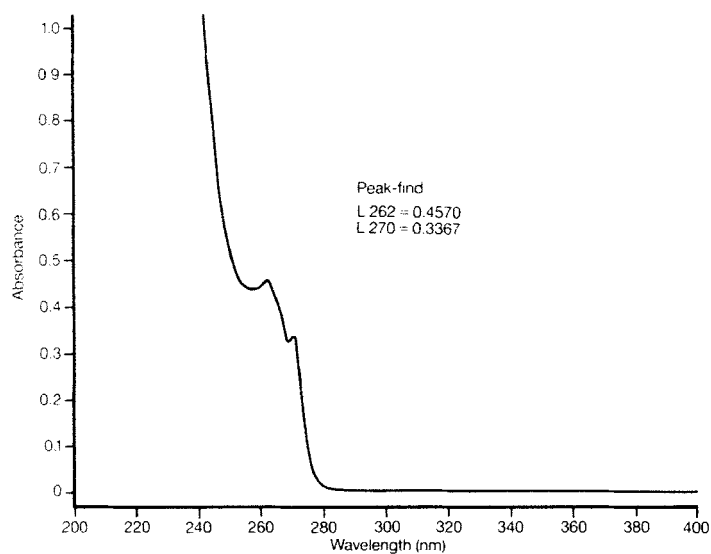


Fig. 8. UV spectrum of lidocaine base in water

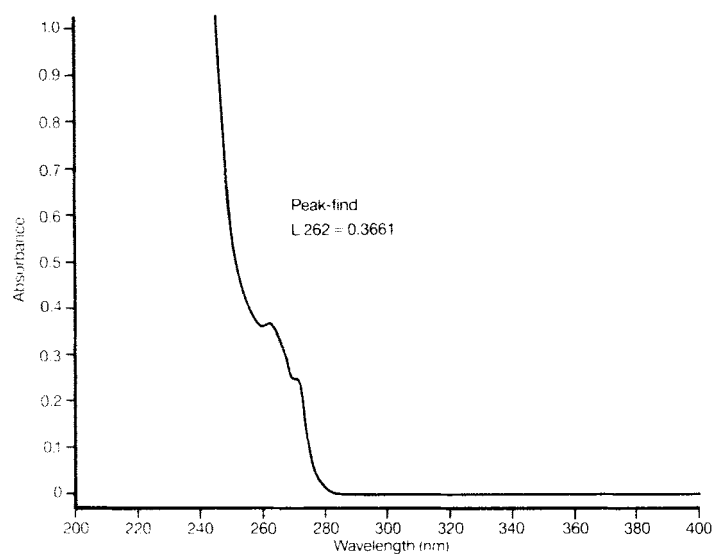


Fig. 9. UV spectrum of lidocaine base in ethanol

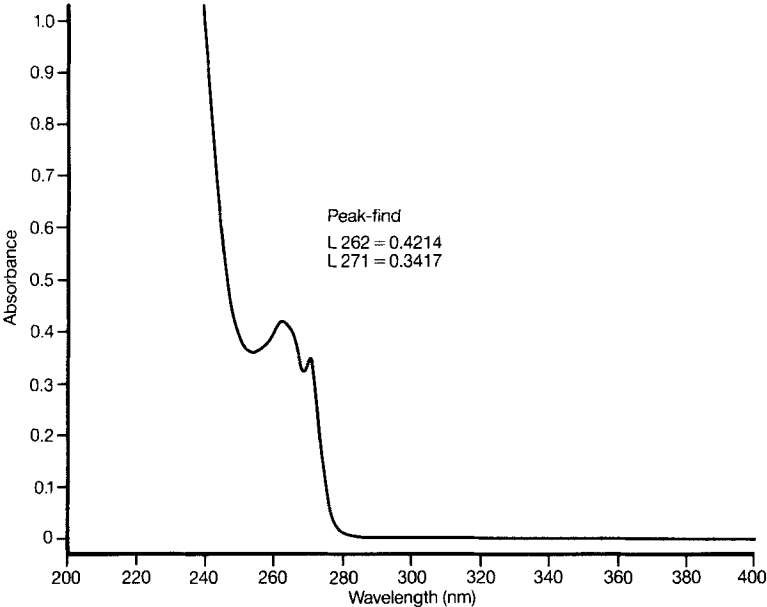


Fig. 10. UV spectrum of lidocaine hydrochloride hydrate in water

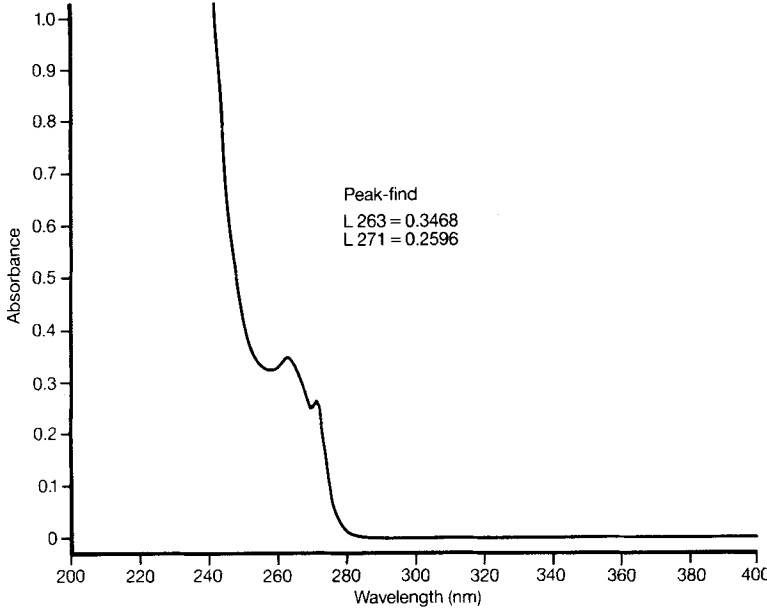
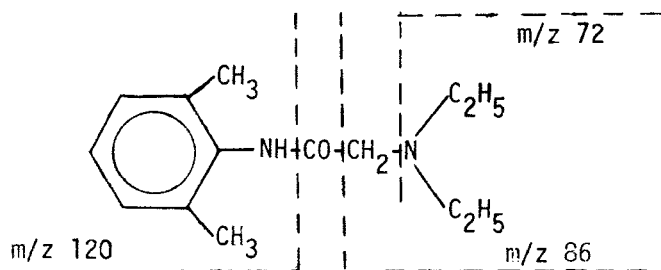


Fig. 11. UV spectrum of lidocaine hydrochloride hydrate in ethanol.

### 3.8 MASS SPECTRUM

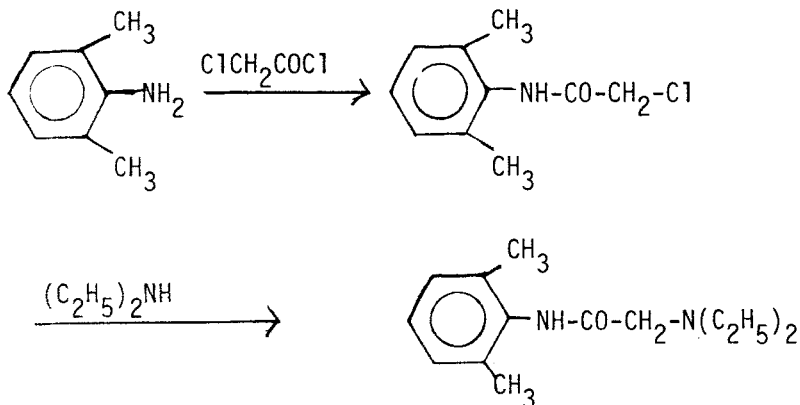
The mass spectrum of lidocaine was obtained with an LKB 2091 gas chromatograph - mass spectrometer (4). It is shown in Figure 12. The fragmentation pattern leading to ions of diagnostic value is outlined below.



$[\text{M}]^+$  is assigned by the ion at  $m/z$  234. The ion at  $m/z$  86 forms the base peak.

### 4. SYNTHESIS

Lidocaine is prepared according to the following scheme:



2,6-Xylidine is acylated with chloroacetyl chloride in the presence of a suitable base, and the resulting xylidide reacted with diethylamine. The product, lidocaine base, is extensively purified and, where appropriate, converted to hydrochloride which is further purified.

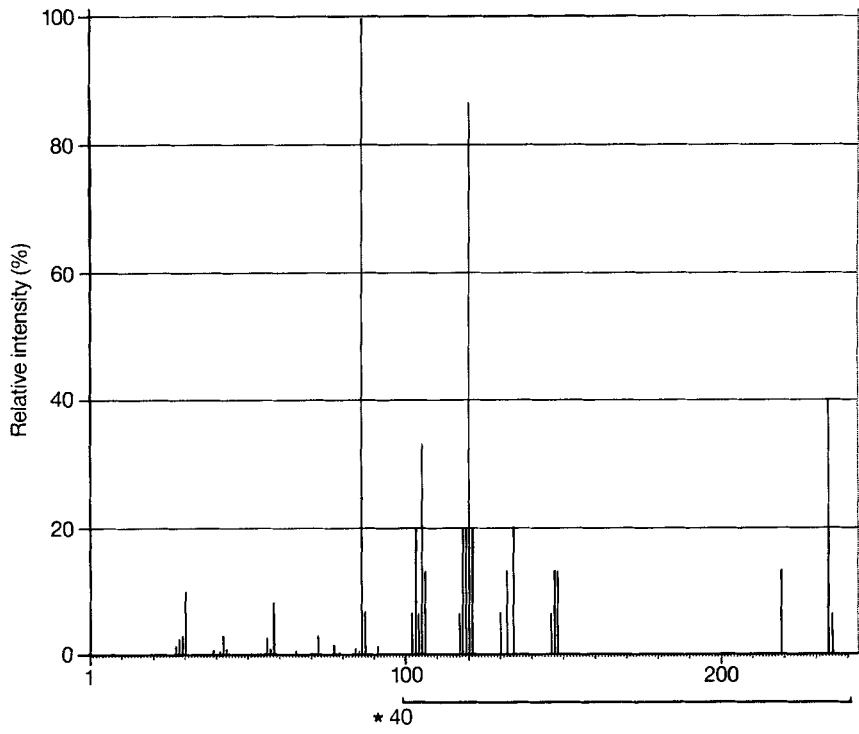


Fig. 12. Mass Spectrum of lidocaine

## 5. METHODS OF ANALYSIS

### 5.1 ELEMENTAL ANALYSIS

The results from elemental analysis are summarized in Table 7.

Table 7. Elemental analysis of lidocaine and lidocaine hydrochloride.

		Theoretical	Found (1)
base (C <sub>14</sub> H <sub>22</sub> N <sub>2</sub> O)	C	71.75 %	71.5 %
	H	9.46 %	9.39%
	N	11.95 %	11.93 %
hydrochloride (C <sub>14</sub> H <sub>23</sub> N <sub>2</sub> OC1)	Cl	13.09 %	13.15 % (Mohr)

### 5.2 IDENTIFICATION TESTS (7,8,9)

The infrared absorption spectrum of a potassium bromide dispersion of the sample (lidocaine base or hydrochloride) exhibits maxima only at the same wavelengths as that of a similar preparation of a chemical reference standard.

To about 0.1 g of lidocaine base dissolved in 1 ml of alcohol is added 10 drops of a 2 %, w/v, aqueous solution of cobaltous chloride containing 1 %, v/v, of hydrochloric acid. The mixture is shaken for about 2 minutes when a bright green colour develops, and a fine precipitate is formed.

To a lidocaine hydrochloride solution (0.2 g in 10 ml of water) is added 10 ml of a 1 %, w/v, picric acid solution. The precipitate, when washed with water and dried, has a melting point of about 230°C.

From lidocaine hydrochloride the base is isolated either by alkalization of an aqueous solution of the salt followed by isolation of the base or by making an alkaline extraction into e.g. chloroform where the base is isolated by evaporation and drying. The identification procedure for the base can then be used.

An aqueous solution of lidocaine hydrochloride yields a white precipitate, insoluble in nitric acid, by addition of silver nitrate. This precipitate is however soluble in a slight excess of ammonium hydroxide (identification of chloride). Another chloride test is performed by mixing equal weights of the sample and manganese dioxide, moistening with sulphuric acid and then heating. Chlorine is then evolved and is recognizable by its odour and by production of a blue colour with moistened starch iodide paper.

### 5.3 TITRIMETRY

Lidocaine base can be determined by dissolving the sample in an excess of 0.1 M hydrochloric acid or 0.05 M sulphuric acid followed by back titration with 0.1 M sodium hydroxide. A mixture of bromocresol green and methyl red is appropriate as indicator (7,10). Non-aqueous titration using a solution of the base in anhydrous glacial acetic acid, crystal violet as indicator, and 0.1 M perchloric acid as titrator is also possible.

For the assay of lidocaine hydrochloride 0.3 g of the sample is dissolved in anhydrous glacial acetic acid, 6 to 7 ml of mercuric acetate solution (7,8,9) is added and the solution is titrated with 0.1 M perchloric acid using crystal violet as indicator. An emerald-green colour indicates the end-point.

Amines and amine salts can be determined by automatic potentiometric two-phase titration (11). Lidocaine hydrochloride (0.2400 g) is weighed into a titration vessel and 20.0 ml of dichloromethane and 20.0 ml of 0.05 M hexadecylpyridinium chloride are added. Titration is performed with 0.1 M sodium hydroxide with vigorous stirring.

### 5.4 GAS CHROMATOGRAPHY

The purity of lidocaine can be determined by gas chromatography using a capillary column of crosslinked SE 54 on fused silica (length 25 m, i.d. 0.3 mm, film thickness 0.5  $\mu\text{m}$ ) and a flame ionization detector. (For instrument settings see Table 8).

The sample (0.20 - 0.25 g of lidocaine) is dissolved in 10 ml of dichloromethane and 2  $\mu\text{l}$  is injected into the gas chromatograph. The chromatogram is evaluated using internal normalization. For determination of lidocaine hydrochloride a base extraction into dichloromethane is performed before injection.

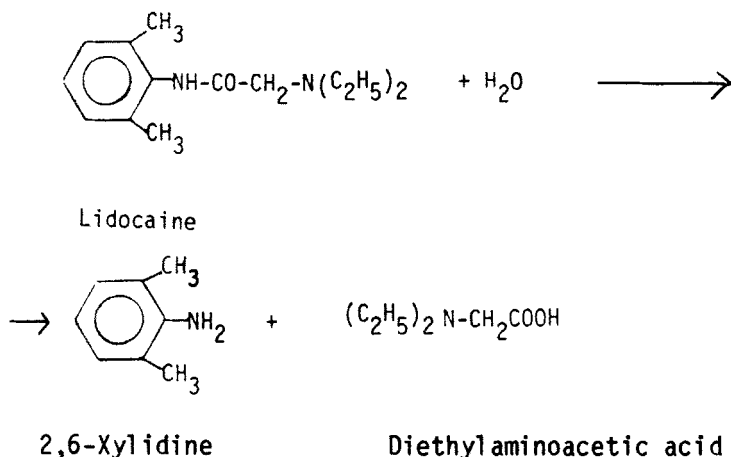


Table 8.

Instrument settings:	Initial oven temperature	140°C
	Initial oven time	4 min.
	program rate	3°C/min.
	Final oven temperature	180°C
	Final oven time	23 min.
	Injector temperature	220°C
	Detector temperature	270°C
	Carrier gas	He
	Inlet pressure	0.7 kg/cm
	Split vent	20 ml/min

## 6. STABILITY - DEGRADATION

In solution lidocaine would be expected to decompose by hydrolysis as follows:



It appears that lidocaine in aqueous solution is extremely resistant to heat, acid and alkali, but when decomposition does occur it is by the hydrolysis as shown above. The high stability is due to the sterical hindrance towards attack on the amido group exhibited by the two ortho methyl groups. However, lidocaine is more readily hydrolysed by acid than by alkali. This can be attributed to the inhibited mesomerism - due to loss of planarity between the benzene nucleus and the amide group - which gives rise to higher electron densities at the amide nitrogen and acyl carbon atoms, thus inhibiting nucleophilic attack. This steric inhibition of mesomerism in lidocaine has been demonstrated by Löfgren (10). Acidic hydrolysis, initiated by protonation of the carbonyl oxygen, should occur somewhat more readily (12).

Stability studies have shown that a 2 per cent solution of lidocaine hydrochloride made alkaline to pH 7.3 and heated in an autoclave at 115°C for 3 hours showed only 0.05 per cent decomposition. By heating in an autoclave with 50 per cent sulphuric acid for 5 hours at 116°C only 3 per cent decomposed while a similar treatment with 20 per cent ethanolic potassium hydroxide solution caused approximately 0.5 per cent decomposition (10). Under more vigorous conditions such as heating for 24 hours in constantly boiling hydrochloric acid solution (108°C, 6.5 M HCl) 50 per cent of the lidocaine remained unhydrolysed.

Solutions for injection containing 1 and 2 per cent lidocaine hydrochloride (pH 6.7) stored for 5 years at room temperature contained only 0.5 µg/ml and 0.7 µg/ml respectively of the hydrolysis product 2,6-xylidine.

## 7. PHARMACOKINETICS

The literature concerning the clinical pharmacokinetics of lidocaine is extensive. The properties of the drug have been summarized in review articles (13-15).

### 7.1 PLASMA CONCENTRATIONS AFTER DIFFERENT ROUTES OF ADMINISTRATION

The major determinants of the systemic absorption of lidocaine are the dose (concentration and volume), the site of injection, the perfusion and tissue binding at the site of injection and whether or not adrenaline has been added. The absorption rate decreases in the order: intercostal block > caudal block > epidural block > brachial plexus block > sciatic and femoral block (14).

The plasma concentrations after endotracheal administration are comparable to those after central and peripheral nerve blocks (16).

Intraperitoneal infusion of 500 to 1000 mg of lidocaine is accompanied by relatively low maximum plasma drug concentrations (17).

Adrenaline reduces the peak plasma concentration of lidocaine at all injection sites but to a varying degree at the different sites (18).

Lidocaine is not used orally due to extensive first-pass metabolism. Observations in patients receiving lidocaine by prolonged infusion for suppression of ventricular dysrhythmias support the generalization that subjective effects are associated with plasma concentrations of 3-5 µg/ml and that objective signs appear at 6-10 µg/ml (15).

## 7.2 PROTEIN BINDING

Lidocaine is bound to plasma proteins to about 60 % at therapeutic drug levels. The fraction bound decreases to about 40 % at 10 µg/ml plasma, which may contribute to the toxicity as the fraction of free drug increases (13,19). Fetal plasma binding is approximately 50 % less than binding in maternal plasma (20). Lidocaine is predominantly bound to  $\alpha_1$ -acid glycoprotein (21).

## 7.3 PLACENTAL TRANSFER

Lidocaine passes the placenta and the rate of diffusion is mainly a function of the concentration gradient between maternal and fetal blood, the pH-gradient between mother and fetus and the maternal protein binding (20,22,23). The ratio between the lidocaine concentrations in the umbilical and maternal vein is 0.52 - 0.69 (24).

## 7.4 BIOTRANSFORMATION AND ELIMINATION

Lidocaine is rapidly and extensively metabolised in man (25,26,27). The major metabolic pathways are N-dealkylation to monoethylglycylxylidide (MEGX) and glycylxylidide (GX), hydroxylation in the aromatic ring and amide hydrolysis (Fig. 13).

The major metabolite in urine is 4-hydroxy-2,6-xylidine. The biological half-life of lidocaine is 1.5 h (13,28). The estimates of plasma clearance range from 0.54 to 1.44 l/min (15) and have been shown to be dependent on liver blood flow (29).

Both MEGX and GX have pharmacologic effects, both as antiarrhythmics and in terms of toxicity (30,31). The half-life of MEGX appears to be about the same as or slightly greater than that of lidocaine, but the potential of MEGX for toxicity would be more apparent in patients with heart failure, in whom its elimination rate is reduced (32,33). GX accumulates in patients with renal failure, which should be considered during continuous administration (34).

The clearance of lidocaine does not differ between elderly (mean age 65 years) and young (mean age 24 years) subjects (35).

Studies in neonates have shown that although the plasma half-life is prolonged, clearance does not differ from that in adults (36). Metabolism of lidocaine by the fetus/neonate has also been confirmed (37).

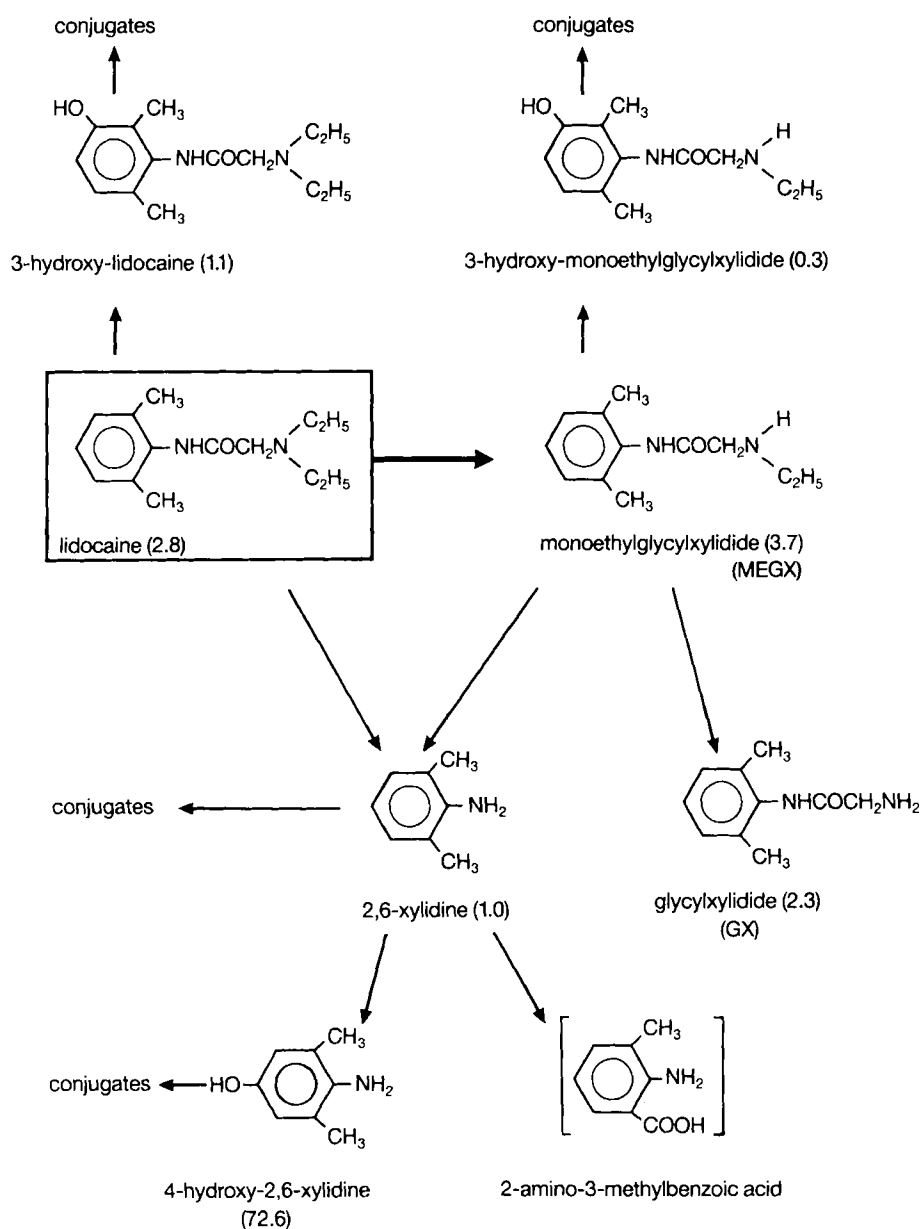


Fig. 13. Pathways for the biotransformation of lidocaine in man. Values in parentheses indicate percentages of dose found in urine.

## 8. METHODS OF ANALYSIS - BIOCHEMICAL APPLICATIONS

### 8.1 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

HPLC is probably the most versatile method for the rapid and specific determination of levels of lidocaine and its active, polar metabolites. Recently, several HPLC methods using ultraviolet (UV) detection have been employed (Table 9) (38-51). Unfortunately, as these compounds contain no chromophore which absorbs strongly in the visible or near-UV regions, the wavelength employed for detection must be approximately 200 nm. In recent years, amperometric electrochemical detection following liquid chromatography has become increasingly popular for the quantitation of easily oxidizable analytes. Its principal advantages include uniformly high sensitivity and a unique selectivity towards compounds that can be electrolyzed at the applied detector potential. The anodic electrochemistry of lidocaine, MEGX and GX at graphite electrodes and the development of an LC-ED procedure for their determination have recently been described (52). The absolute detection limits for these compounds were 2 ng, 5 ng and 4 ng injected, respectively.

### 8.2 GAS CHROMATOGRAPHY (GC)

Numerous gas chromatographic approaches have been described for monitoring of lidocaine alone or together with its metabolites MEGX and GX (Table 10), (53-76). These methods, employing flame ionization or nitrogen-phosphorous detection have been shown to possess excellent sensitivity for lidocaine, generally in the 1-10 ng/ml range. But, in many procedures, the metabolites MEGX and GX either are not determined at all or are not separately distinguished from the parent compound. More importantly, the application of all these approaches to the routine analysis of real serum samples is limited by the sample preparation time taken up by the lengthy derivatization, extraction, or evaporation/preconcentration procedures required.

### 8.3 GAS CHROMATOGRAPHY - MASS SPECTROMETRY (GC/MS), SELECTED ION MONITORING

As proposed in ref 77 the term "selected ion monitoring" is used. In this application the mass spectrometer is used to monitor ions of certain mass normally in the gas chromatographic effluent. The technique is also commonly referred to as "mass fragmentography" in addition to various other names.

Table 9. HPLC methods for measuring lidocaine and metabolites in biological material

Sample	Internal standard	Extracting solvent	Detector	Analytical precision (CV %)	Metabolites measured	Ref
Serum	Procaine	Charcoal adsorption	UV	6-10	No	38
Plasma	Bupivacaine	Ethyl acetate	UV	-	No	39
Serum Urine	Ethylmethylglycylxylidide (EMGX)	Chloroform-hexane - isopropanol	UV	0.9 - 5	MEGX GX	40
Plasma	None	Methylene chloride	UV	< 10	No	41
Plasma	None	None	UV	-	MEGX GX	42
Plasma	EMGX	Ethyl acetate	UV	2-4	MEGX GX	43
Serum	P-Chlorodisopyramide	Dichloromethane	UV	< 5	No	44
Plasma	EMGX	Methylene chloride	UV	< 7	MEGX GX	45
Serum CSF	Mepivacaine	Chloroform-hexane-isopropanol	UV	3-5	MEGX GX	46-47
Serum Urine	2-Amino-N-(2,6-xyllyl)-butyramide	Ethyl acetate	UV	2-7	MEGX GX	48
Plasma Myocardial samples (rabbit)	EMGX	Ethyl acetate	UV	-	MEGX GX	49
Serum	EMGX	Chloroform	UV	< 2	MEGX GX	50
Plasma	EMGX	Dichloromethane	UV	< 5	MEGX GX	51
Serum	Bupivacaine	Sep-Pak	Electrochemical detection	5-10	MEGX GX	52

Table 10 Gas chromatographic methods for measuring lidocaine and metabolites in biological material

Sample	Internal standard	Column	Detector	Derivative	Analytical precision (CV %)	Metabolites measured	Ref
Blood	Methadone	Packed	FID	None	5	No	53
Plasma	Cyclizine	Packed	NPD	None	-	No	54
Plasma	Aceto-p-	Packed	NPD	Acetyl-derivative	3-8	MEGX	55
Urine	-toluidide					GX 4-hydroxy-2,6-xylidine	
Plasma	Pyrocaine	Packed	NPD	None	2-18	MEGX	56
Plasma	EMGX	Packed	NPD	None	-	No	57
Plasma	Benzhexol	Packed	FID	None	1-7	MEGX	58
Tissue	Mepivacaine	Packed	FID	None	-	No	59
Plasma	Aminopyrine	Packed	FID	None	-	No	60
Plasma	Mepivacaine	Packed	FID	None	6	No	61
Plasma	Trimecaine	Capillary	NPD	Trifluoro-acetyl derivative	4-7	MEGX GX	62
Blood	Mepivacaine	Packed	FID	None	2	No	63
Plasma							
Blood	N-Methylhexo-	Capillary	NPD	None	-	No	64-65
Plasma	barbital						
Blood	Eicosane	Packed	FID	None	2-6	No	66
Plasma							
Various biological fluids	Mepivacaine	Packed	NPD	None	-	No	67
Plasma	Eicosane	Packed	FID	None	-	No	68
Plasma	2-Chloro-6-methylphenyl-acetamide	Packed	FID	None	-	No	69
Urine (horse)							
Plasma	Mepivacaine	Packed	FID	None	<5	No	70
Plasma	Mepivacaine	Packed	NPD	none	1-7	MEGX GX	71
Plasma (cat)	Mepivacaine	Packed	FID	None	2	No	72
Plasma	Ethidocaine	Packed	FID	None	<5	No	73
Post mortem fluids and tissues	SKF 525A	Packed	NPD	None	3	MEGX	74
Blood	Mepivacaine	Packed	NPD	None	3	No	75
Plasma							
Plasma	EMGX	Packed	NPD	None	1-17	MEGX GX	76

In three studies (78, 79, 81) the gas chromatographic peaks of MEGX and GX were asymmetrical and the lower limit of analytical sensitivity for both metabolites was about 0.5 µg/ml. Another mass spectral method based on earlier work using stable isotope labeling and chemical ionization procedures involves the use of deuterated lidocaine MEGX and GX as internal standards (80). Samples containing these standards are extracted and then analyzed by direct probe introduction and isobutane chemical ionization mass spectrometry. The range of detection was from 5 ng to 4 µg for lidocaine and 0.1 µg to 1 µg for MEGX. GX could not be accurately quantified due to interfering substances.

A method using the propyl derivatives of MEGX and GX formed directly in either plasma or urine by treatment with propionaldehyde and sodium cyanoborohydride has been published (82). The derivatives and unchanged lidocaine are extracted, separated by gas chromatography and quantitated by selected ion monitoring, using mepivacaine as the internal standard. Quantitation of these compounds to levels as low as 50 ng/ml body fluid has been achieved with a coefficient of variation less than 10 % (Table 11).

Table 11 Selected ion monitoring methods for measuring lidocaine and metabolites in biological material

Sample	Internal standard	Technique	Derivative	Analytical precision (CV %)	Metabolites measured	Ref
Plasma	Trimecaine	Electron impact	None	3-7	MEGX	78
Plasma Urine	Trimecaine	Electron impact	None	3-9	MEGX GX	79
Plasma	<sup>2</sup> H <sub>4</sub> -Lidocaine <sup>2</sup> H <sub>3</sub> -MEGX	Isobutane chemical ionization Direct inter-tion probe	None	-	MEGX	80
Plasma Urine	Trimecaine 2,4,6-Trimethyl-aniline 2-Napthol	Electron impact	None	3-9	MEGX GX 4-hydroxy-2,6-xyllidine	81
Plasma Urine	Mepivacaine	Electron impact	Propyl derivative	4-10	MEGX GX	82
Blood Urine Tissue	SKF-525A	Electron impact	None	3-6	MEGX	74



## 8.4 HOMOGENEOUS ENZYME IMMUNOASSAY, EMIT<sup>®</sup>

EMIT<sup>®</sup> (Syva Corp. Palo Alto, CA 94304) is a nonisotopic technique that is used to measure lidocaine. Briefly, the procedure is based on competitive protein binding with glucose-6-phosphase dehydrogenase to label the drug and antibody to lidocaine as the specific binding protein. The enzyme activity, monitored as a change in absorbance at 340 nm due to conversion of NAD<sup>+</sup> to NADH, correlates directly with the concentration of lidocaine in the sample. This technique is simple and very rapid and requires only 50 µl of serum. However, since chromatography is not involved, interactions can occur with the antibody and cause falsely elevated drug levels.

Several studies have been performed to evaluate the accuracy and sensitivity of the EMIT<sup>®</sup> lidocaine assay by comparing it with the results obtained by gas chromatographic methods (83 - 86).

The technique has also been used for investigation of the importance of blood-collection tubes in plasma lidocaine determinations (87), antibody selectivity (88) curve stability (89) and pharmacokinetics in thermally injured rats (90). Recently a procedure for adapting the EMIT<sup>®</sup> procedure to the Cobas Bio centrifugal analyzer has been described which avoids the decrease in precision at the upper end of the therapeutic range reported in other adaptations (91). The latest progress in the area of enzyme immunoassay techniques have appeared in several abstracts from the National meetings for the American Association for Clinical Chemistry (92 - 97).

## 9. IDENTIFICATION AND DETERMINATION IN PHARMACEUTICALS

### 9.1 IDENTIFICATION TESTS

A sample (alkalized if containing lidocaine hydrochloride) is extracted with n-hexane or chloroform followed by evaporation to dryness. An infrared absorption spectrum in a potassium bromide dispersion of the residue exhibits maxima only at the same wavelengths as that of a similar preparation of a chemical reference standard.

Apart from infrared spectroscopy the additional identification test presented in Section 5.2 may be performed on the extraction residue.

Comparison of retention data (e.g. retention times in gas chromatography and liquid chromatography and  $R_f$ -values in thin-layer chromatography (98)) with those of a reference standard may serve as identity. In addition, when using liquid chromatography and a variable wavelength detector, absorption ratios between two wavelengths (e.g. 254 nm and 265 nm) are valuable contributions to identity.

## 9.2 EXTRACTION ANALYSIS

To an aliquot (not more than 30 ml) of injection solution corresponding to about 0.2 g of lidocaine hydrochloride is added 2 ml of 2 M sodium hydroxide. The solution is extracted with two 20-ml portions of chloroform. To the combined chloroform extracts are added 10 ml of dioxane and five drops of a mixed indicator (2 parts of 0.2 %, w/v, BZL-blue and 3 parts of 0.1 % w/v, alcoholic solution of methyl red). Titration is performed with 0.1 M perchloric acid to a reddish-violet endpoint.

Lidocaine hydrochloride in Lidocaine Injection and Jelly and Lidocaine in Lidocaine Ointment are determined by base extraction of a diluted sample with chloroform. After evaporation of the combined chloroform extracts, sulphuric acid in excess is added. The excess is titrated with sodium hydroxide using a potentiometric determination of the endpoint (7).

The acid-dye technique has been used for Injections and Creams containing Lidocaine (99). The sample, buffered to pH 3.6, is extracted with chloroform using the ion-pairing dye reagent bromophenol blue. The chloroform phase is rendered alkaline and the blue colour is measured photometrically at 600 nm.

## 9.3 GAS CHROMATOGRAPHY

Determination of lidocaine, after removal from the sample matrix by base extraction e.g. into dichloromethane, can be performed by gas chromatography preferably using mepivacaine as internal standard. The chromatographic system may be identical to that presented in Section 5.4, i.e. using a capillary column and flame ionization detection. Additional columns that have been used contain 3 % OV-17 on 80 - 100 mesh Chromosorb W HP (detector and injector temperatures 250°C, column temperature 190°C) (72) and 5 % OV-101 on 80 - 100 mesh Chromosorb W HP (detector temperature 275°C, injector temperature 310°C, column temperature 190°C) (71).

#### 9.4 CAPILLARY ISOTACHOPHORESIS

Capillary isotachophoresis has been used for the qualitative and quantitative simultaneous determination of lidocaine-, procaine- and tetracaine hydrochlorides in drugs (100). The recovery of the method is about 99.7 % and acids, vitamins and nonionised organic compounds present in the drugs do not interfere with the isotachophoretic separation.

#### 9.5 LIQUID CHROMATOGRAPHY

Liquid chromatography (HPLC) is the most widely used technique for determination of lidocaine in pharmaceuticals. The chromatography has been performed in straight-phase as well as in reversed-phase modes using UV-detection at 230 - 254 nm.

Suitable chromatographic systems are given in Table 12.

#### ACKNOWLEDGEMENT

We wish to express our sincere thanks to Mrs Christin Andersson for her assistance in the preparation of the manuscript.

Table 12 Liquid Chromatographic Systems for Lidocaine

Column	Eluent	Flow rate (ml/min)	Retention time (min)	Ref
Nucleosil 5 NO <sub>2</sub> , dp = 5 $\mu$ m (Macherey-Nagel) 200 x 4 mm i.d.	n-Hexane - ethanol (97:3)	1.0	8.1	4
Nucleosil 7 OH, dp = 7.5 $\mu$ m (Macherey-Nagel) 200 mm x 4 mm i.d.	n-Hexane - ethanol (97:3)	1.0	6.9	4
MicroPak-CN-10, dp = 10 $\mu$ m (Varian) 250 x 2.1 mm i.d.	n-Heptane - dichloromethane- acetonitrile - propylamine (50 : 75 : 20 : 0.1)	1.0	3.6	101
MicroPak-CN-10, dp = 10 $\mu$ m (Varian) 250 x 2.1 mm i.d.	n-Heptane - dichloromethane- acetonitrile - propylamine (50 : 75 : 5 : 0.1)	1.0	4.4	101
$\mu$ Bondapak CN, dp = 10 $\mu$ m (Waters) 300 x 4 mm i.d.	Acetonitrile - phosphate buffer pH 3.0, $\mu$ = 0.1, containing 0.1 % w/v, 1-octanesulphonic acid, sodium salt (10 : 90)	0.8	7.9	4
$\mu$ Bondapak CN, dp = 10 $\mu$ m (Waters) 300 x 4 mm i.d.	Acc to USP XX, Third Supple- ment	1.5	-	7
$\mu$ Bondapak CN, dp = 10 $\mu$ m (Waters) 300 x 4 mm i.d.	0.01 M 1-Octanesulphonic acid, 2.0 sodium salt, 2 % v/v, acetic acid, 2 % v/v, acetonitrile, and 1 % v/v, methanol in water		6.8	102
Nucleosil C <sub>18</sub> , dp = 10 $\mu$ m (Macherey-Nagel) 300 x 4 mm i.d.	Tetrahydrofuran - 50 mmol/l phosphate buffer adjusted to pH 3.5 ( 3 : 97 )	2.0	5.6	46
LiChrosorb RP-8 dp = 10 $\mu$ m (Merck) 200 x 3 mm i.d.	Acetonitrile- phosphate buffer pH 8.0 ( $\mu$ = 0.5). ( 600 : 400)	1.0	3.1	4
LiChrosorb RP-8, dp = 5 $\mu$ m, Hiber <sup>R</sup> (Merck) 125 x 4 mm i.d.	Acetonitrile- phosphate buffer pH 8.0, $\mu$ = 0.05 (600 : 400)	1.0	3.0	4
Spheri 10 RP 8 (Brownlee Labs) 100 x 4.6 mm i.d. with Spheri 10 RP 8, 30 x 4.6 mm i.d., guard column	Acetonitrile- phosphate buffer pH 8.0, $\mu$ = 0.05, ( 500 : 500)	0.8	6.5	4
Octadecyl silane, chemically bonded to silica	Phosphate buffer pH 7.0 - water - acetonitrile (50 : 400 : 550)	2.0	-	7
Nova Pak C <sub>18</sub> , dp = 5 $\mu$ m (Waters) 150 x 4 mm i.d.	Methanol- phosphate buffer pH 8.0 $\mu$ = 0.05 ( 700 : 300)	1.0	3.5	4
$\mu$ Bondapak C <sub>18</sub> , dp = 10 $\mu$ m (Waters) 300 x 4 mm i.d.	Acetonitrile - phosphate buffer pH 8.0, $\mu$ = 0.05 (600 : 400)	1.4	4.2	4
$\mu$ Bondapak C <sub>18</sub> , dp = 10 $\mu$ m (Waters) 200 x 3 mm i.d.	Acetonitrile - phosphate buffer pH 6.2, $\mu$ = 0.05, containing $2.9 \times 10^{-4}$ M tetrapentylammonium- chloride (200 : 800)	1.4	4.1	4
$\mu$ Bondapak C <sub>18</sub> , dp = 10 $\mu$ m (Waters) 150 x 4 mm i.d.	Acetonitrile - phosphate buffer pH 6.2, $\mu$ = 0.05, containing $2.9 \times 10^{-3}$ M tetrabutylammonium- hydrogensulphate (200 : 800)	1.6	5.5	4

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# BENPERIDOL

Pamela Girgis Takla, Kenneth C. James  
and Ahmed Eliass H. Gassim

*The Welsh School of Pharmacy  
Cardiff, Wales*

1. Description	245
1.1 Name, Formula, Molecular Weight	245
1.2 Appearance, Colour, Odour	245
1.3 Therapeutic Category	245
1.4 Usual Dose Range	245
2. Physical Properties	246
2.1 Infrared Spectra	246
2.2 Nuclear Magnetic Resonance Spectrum	246
2.3 Ultraviolet Absorption Spectrum	250
2.4 Mass Spectrum	253
2.5 Melting Range	256
2.6 Differential Scanning Calorimetry	256
2.7 Solubilities	257
2.8 pKa Values	258
2.9 X-Ray Diffraction	259
3. Synthesis	259
4. Drug Metabolism and Pharmacokinetics	261
5. Stability, Degradation, and Purity Determination	263
6. Methods of Analysis	265
6.1 Polarography	265
6.2 Fluorimetric Analysis	265
6.3 Chromatography	266
7. Identification and Determination in Pharmaceuticals	267
8. Identification and Determination in Body Fluids	268
8.1 Identification	268
8.2 Chromatography	269
References	270

## 1. Description

### 1.1 Name, Formula, Molecular Weight

Benperidol is 1-[1-[4-(4-fluorophenyl)-4-oxobutyl]-4-piperidiny]-1,3-dihydro-2H-benzimidazol-2-one. [CAS Registry Number: 2062-84-2].

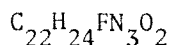
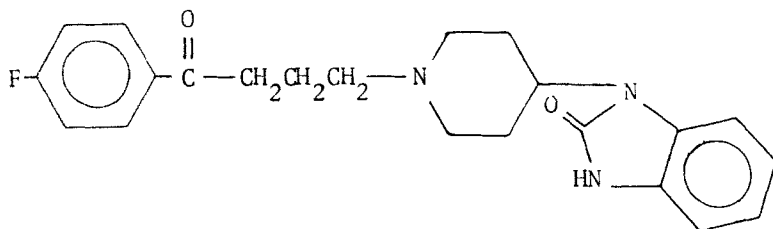
Other chemical names by which it has also been known include:

1-[1-(3-p-fluorobenzoylpropyl)piperid-4-yl]benzimidazolin-2-one;<sup>1</sup>

1-[1-[4-(p-fluorophenyl)-4-oxobutyl]piperidin-4-yl]-2-benzimidazolinone<sup>2</sup>.

Synonyms: Benzperidol; CB 8089; McN-JR 4584; R 4584.

Proprietary Names: Anquil; Frenactil; Glianimon; Concilium.



Molecular Weight = 381.5

### 1.2 Appearance, Colour, Odour<sup>1</sup>

Odourless, off-white, amorphous or crystalline powder which darkens slowly on exposure to light.

### 1.3 Therapeutic Category

Tranquilliser.

### 1.4 Usual Dose Range<sup>1</sup>

Up to 6 mg daily.

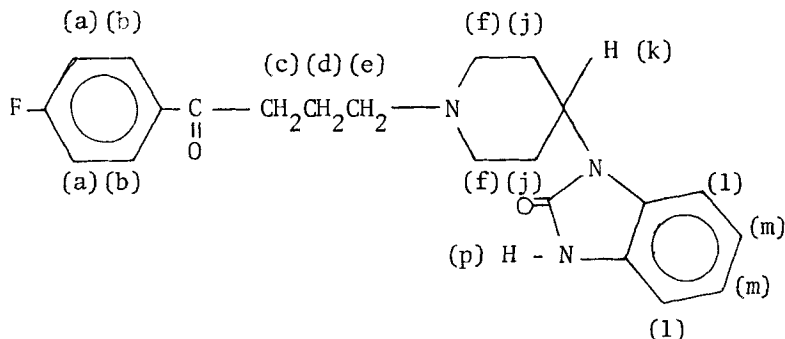
## 2. Physical Properties

### 2.1 Infrared Spectra

Fig. 1 shows the infrared spectrum of a sample of benperidol, supplied by Janssen Pharmaceutical Limited, recorded from a potassium bromide disc using a Perkin-Elmer Model 681 infrared spectrophotometer. Bands in the spectrum correspond to peak assignments made by Azibi et al.<sup>3</sup> for a spectrum (which was not published) obtained for benperidol crystals, designated polymorphic form I. These assignments are given in Table I, with an added assignment for the weak absorption band at  $3410\text{ cm}^{-1}$  as an overtone of the carbonyl stretching vibration at  $1710\text{ cm}^{-1}$ .

Figs. 2-6 and Table II show infrared spectra and peak assignments for other forms of benperidol prepared and characterised as described by Azibi et al.<sup>3</sup> These include two polymorphic forms (Figs. 2 and 3) obtained by re-crystallisation from isopropanol and n-heptane respectively, the ethanolate (Fig. 4), dihydrate (Fig. 5) and amorphous forms (Fig. 6). The ethanolate was prepared by dissolving the commercial (anhydrous) form in ethanol previously heated to  $50^\circ$ , and allowing the solution to crystallise slowly. Re-crystallisation from a mixture of equal volumes of water and acetone was carried out to produce a dihydrate (Fig. 5). The amorphous form (Fig. 6) was produced when the commercial product was melted, and then allowed to solidify at room temperature. The peak assignments given in Table II are similar to those reported by Azibi et al.<sup>3</sup>, and the spectra shown for the ethanolate and the hydrate are identical to their published spectra. The paper of Azibi et al. did not include spectra for the other forms.

### 2.2 Nuclear Magnetic Resonance Spectrum



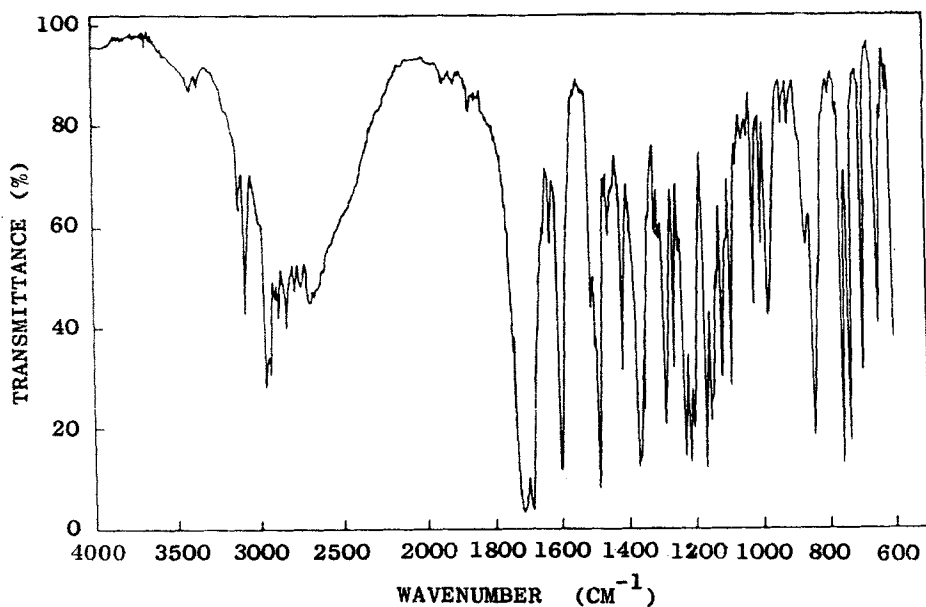


Figure 1. Infrared spectrum of a commercial sample of benperidol (polymorphic form I) - KBr disc.

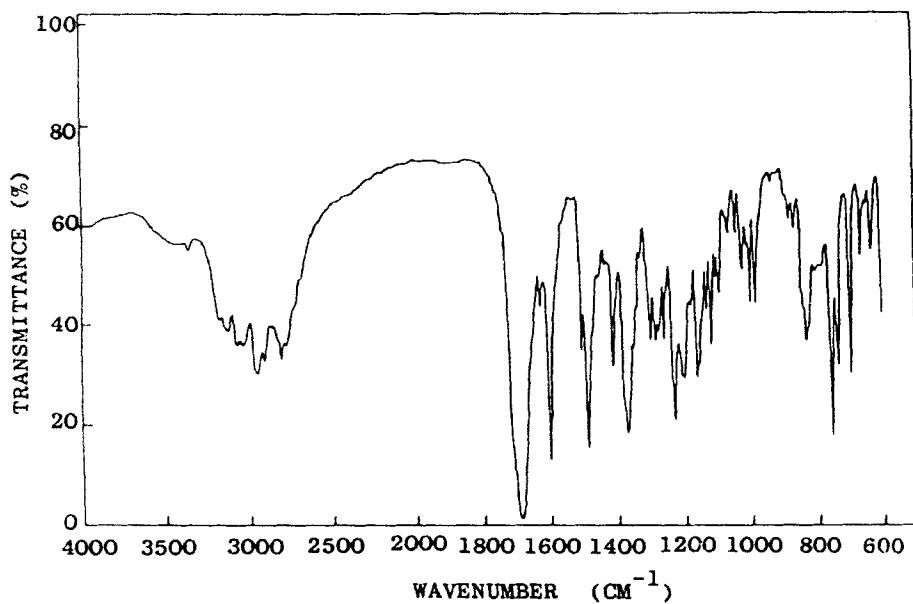


Figure 2. Infrared spectrum of benperidol re-crystallised from isopropanol - KBr disc.

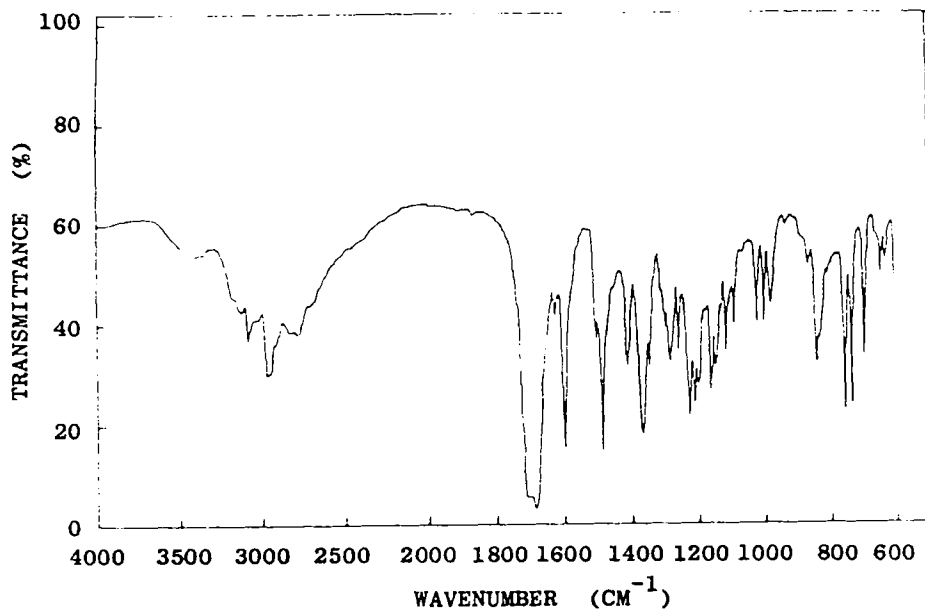


Figure 3. Infrared spectrum of benperidol re-crystallised from n-heptane - KBr disc.

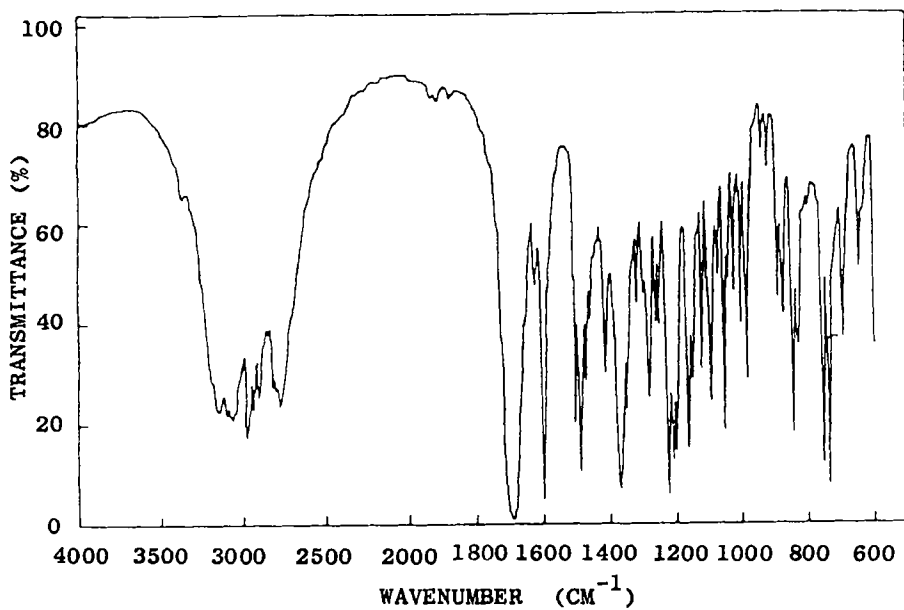


Figure 4. Infrared spectrum of benperidol ethanolate - KBr disc.



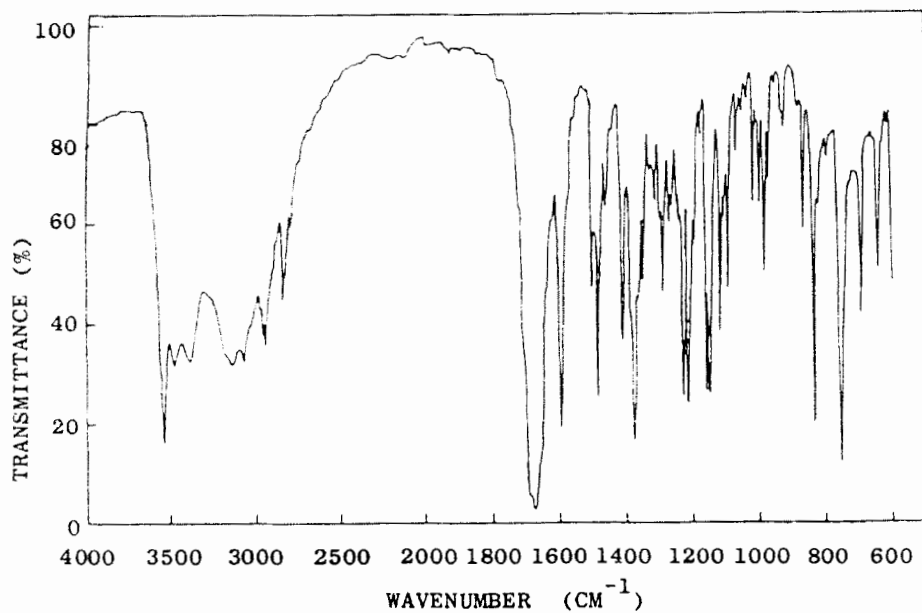


Figure 5. Infrared spectrum of benperidol dihydrate  
- KBr disc.

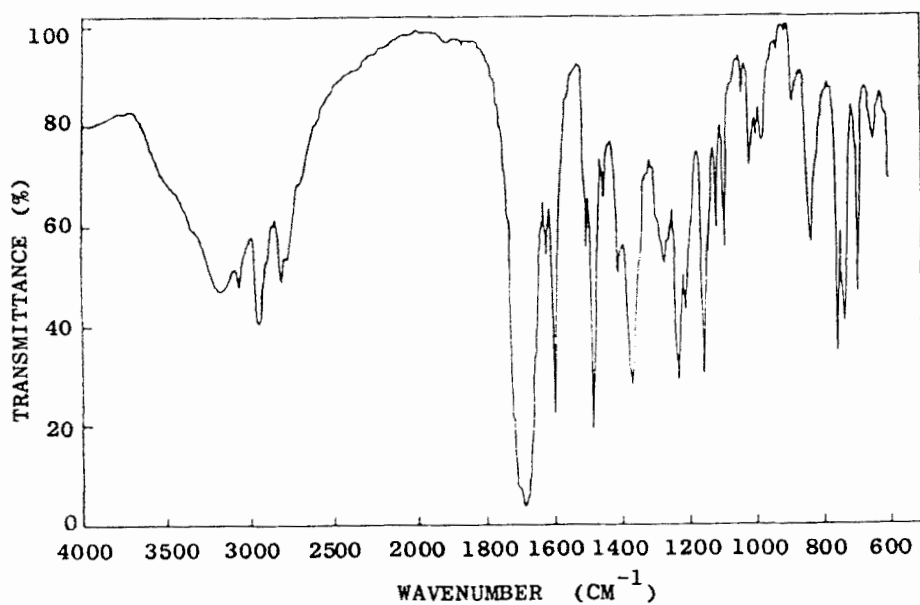


Figure 6. Infrared spectrum of amorphous benperidol  
- KBr disc.

The 90 MHz spectrum of benperidol, shown in Fig. 7, was obtained in deuterated dimethylsulphoxide at a concentration of 80 mg/ml using a Perkin-Elmer R32 spectrometer. Tetramethylsilane was used as the reference standard. Table III shows the spectral assignments.

Table I

<u>Infrared Spectral Assignments for Commercial Sample of Benperidol</u>		
<u>Wave number, cm<sup>-1</sup></u>		<u>Assignment</u>
3410, 3360	2	Overtones of C=O stretching frequencies
3110		N-H stretch
3080, 3030		Aryl =C-H stretch
2960, 2930, 2900, 2880		-CH <sub>2</sub> - stretch
2830, 2780, 2760, 2700		-CH <sub>2</sub> - stretch of groupings bonded to nitrogen of piperidine ring
1710		C=O stretch (benzimidazole)
1685		C=O stretch (fluorophenyl)
1595, 1505, 1485, 1455, 1415		Aromatic C=C i.p. stretch
1365, 1345		Methylenic -CH <sub>2</sub> - deformation bands
1225		C-F stretch
1165, 1140		Aryl C-H i.p. deformation
840		Aryl C-H o.p. deformation (fluorophenyl ring) (1:4 disubstitution)
755, 730		Aryl C-H o.p. deformation (benzimidazole) (1:2 disubstitution)

### 2.3 Ultraviolet Absorption Spectrum

In 0.1 M hydrochloric acid:isopropanol (1:9), benperidol has been reported to show maxima at 232 nm (A 1%, 1cm 349), 243 nm (A 1%, 1cm 354), and 297 nm (A 1%, 1cm 185)<sup>1</sup>.

Spectra were determined in other solvents using 1 cm silica cells with a Pye-Unicam SP 1800 spectrophotometer. A Pye-Unicam SP 500 Series 2 spectrophotometer was used for measuring absorbances at the wavelength maxima. The benperidol sample used showed not more than a 0.04% loss of weight on drying to constant weight in vacuo at 60°. The solvent mixture specified for measuring the ultraviolet absorption of haloperidol in the British Pharmacopoeia<sup>4</sup> is 0.1 M hydrochloric acid:methanol (10 volumes:90 volumes). In this mixture, benperidol showed maxima at 233 nm (A 1%, 1cm 337), 244 nm (A 1%, 1cm 352) and 281 nm (A 1%, 1cm 188).

Table II

Infrared Spectral Assignments for Different Forms of Benperidol Prepared from the Commercial Sample (Form I)

	Polymorph (from iso- propanol)	Polymorph (from n- heptane)	Ethano- late	Dihydrate	Amor- phous Form
OH			3150	3540, 3480	
C=O $\nu$ overtone	3360	3360	3370	3380	3360 (shoulder)
N-H $\nu$	3180, 3140	3180, 3120	3100	3140	3180
Aryl =C-H $\nu$	3080, 3040 3030	3080, 3050 3020	3080, 3060 3040	3070, 3020	3070
-CH <sub>2</sub> - $\nu$	2950, 2910	2960, 2930	2980, 2905 2940	2960, 2930 2900, 2880	2950, 2900
-CH <sub>2</sub> - $\nu$ (adj. pip- eridine N)	2810, 2770	2800, 2770	2850, 2820 2800, 2780	2880, 2840 2800, 2740	2810, 2780
C=O $\nu$	1690	1710, 1685	1690	1680	1690
Aryl C=C i.p. $\nu$	1600, 1505 1488, 1470 1400	1595, 1505 1485, 1470 1410	1600, 1505 1490, 1470 1410	1595, 1505 1482, 1450 1410	1600, 1505, 1485, 1450, 1410
-CH <sub>2</sub> - def.	1370	1370, 1350	1370, 1350	1375, 1348	1370
C-F $\nu$ (shoulder)	1225	1225	1223	1230	1230
Aryl C-H i.p. def.	1160	1165, 1150	1160, 1150	1160, 1148	1155
Aryl C-H o.p. def. 1:4 disub.	830	830 (shoulder)	843	835	835
Aryl C-H o.p. def. 1:2 disub.	758, 735	755, 735	753, 735	755	755, 735

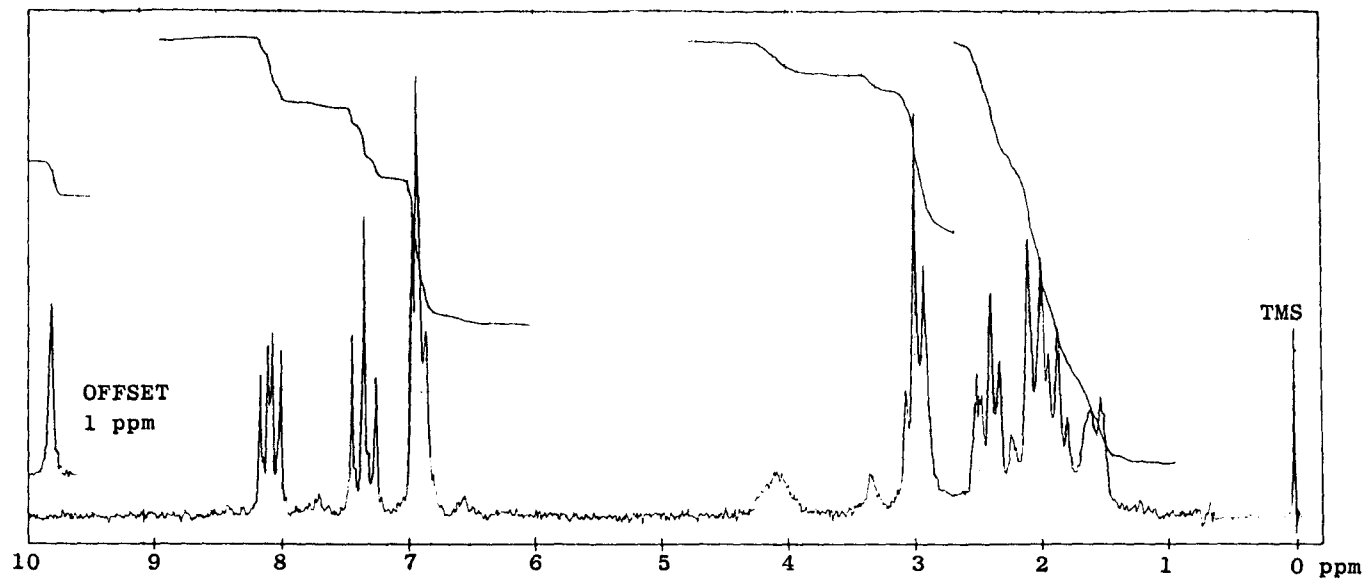


Figure 7. Nuclear magnetic resonance spectrum of benperidol in dimethylsulphoxide-D<sub>6</sub>.

In 0.1 M hydrochloric acid:methanol (90 volumes:10 volumes), used for haloperidol by Janicki and Ko<sup>5</sup>, benperidol showed maxima at 231 nm (A 1%, 1cm 286), 248 nm (A 1%, 1cm 338) and 278 nm (A 1%, 1cm 204). The spectra obtained in these hydrochloric acid:methanol mixtures are shown in Fig. 8.

Table III  
NMR Spectral Assignments for Benperidol

Chemical Shift (ppm)	Number of Protons	Multiplicity	Characteristic of Proton
10.8	1	singlet	p
8.1	2	multiplet	b
7.35	2	triplet	a
6.9	4	two overlapping doublets	l,m
4.1	1	broad multiplet	k
3.0	4	two overlapping triplets	c,e
2.4-1.6	10	multiplet	d,f,j

#### 2.4 Mass Spectrum

The mass spectral fragmentation pattern of benperidol has been discussed by Blessington<sup>6</sup>, who obtained a spectrum with an AEI MS-902 instrument, and also by Leferink and Maes<sup>7</sup> who obtained electron impact (EI) and chemical ionisation (CI) mass spectra of benperidol and some other butyrophenones with isobutane as the reagent gas, using a Finnigan Model 1015-D mass spectrometer. The mass spectrum of benperidol as the free base, or as a salt such as the picrate, is said to provide a sensitive and specific means for its identification and quantitation in pharmaceutical or biological materials.

EI fragmentation pathways for benperidol are shown below, the numbers in parentheses representing relative intensities.

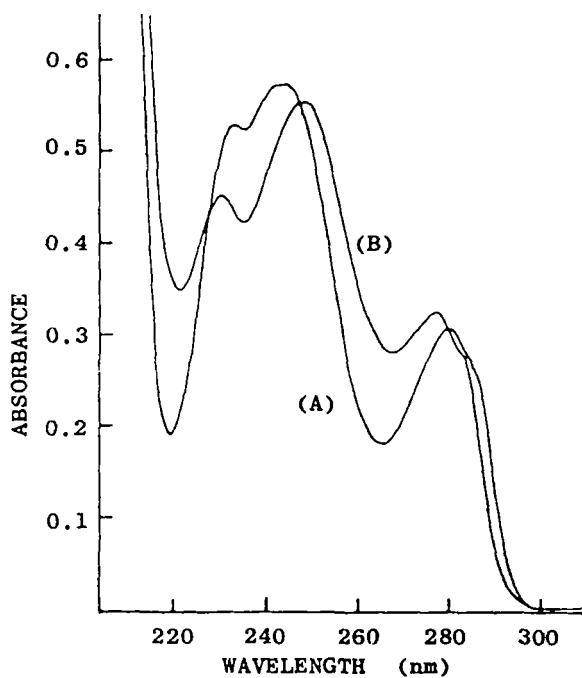
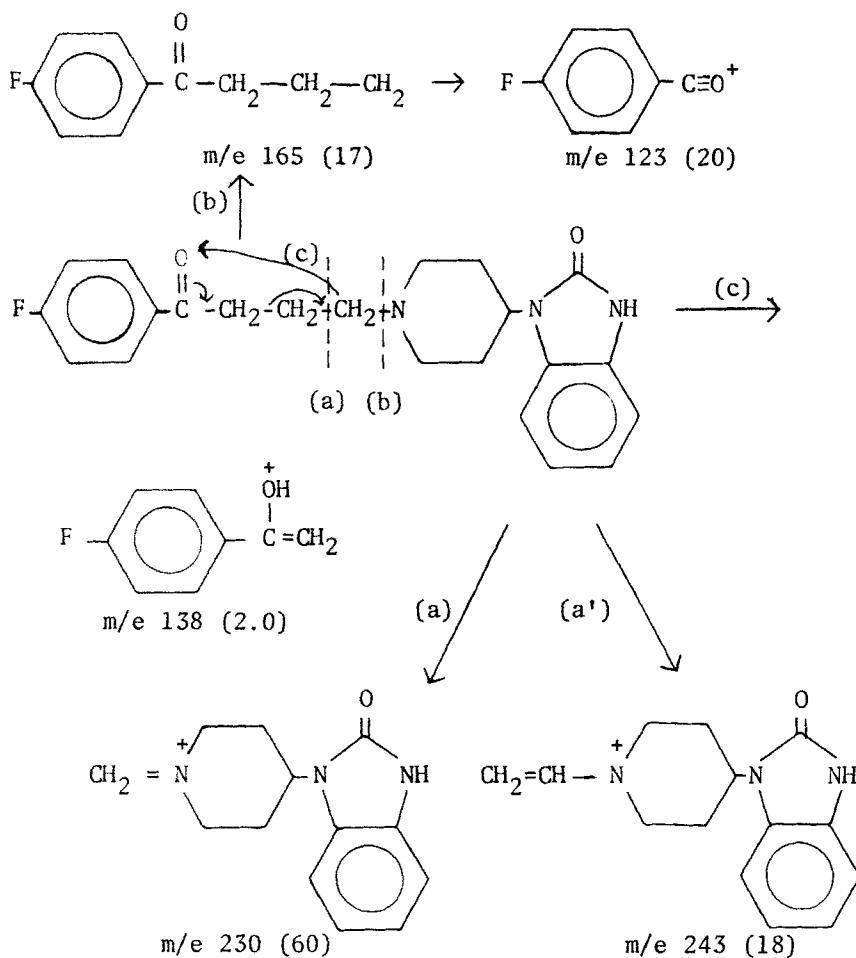


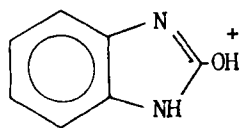
Figure 8. Ultraviolet absorption spectra for  $4.19 \times 10^{-5}$  M benperidol in 0.1 M hydrochloric acid : methanol (A) 10 volumes : 90 volumes; and (B) 90 volumes : 10 volumes.



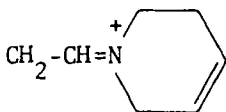
Ions are formed by fragmentation pathway (a) through a simple cleavage of the butyrophenone chain, directed by an ion radical sited on the tertiary nitrogen of the piperidine ring. The ions formed by fragmentation pathway (a') are thirteen mass units higher than those formed by pathway (a), and can be accounted for by a McLafferty type fragmentation of the butyrophenone chain with charge retention on the nitrogen fragment.

Fragments containing the p-fluorobenzoyl moiety exhibit peaks at  $\text{m/e 165}$ ,  $\text{123}$  and  $\text{95}$ . The latter arises by loss of CO from the  $\text{m/e 123}$  ion.

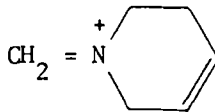
Other important fragment ions are:



m/e 134 (10)



m/e 109 (100)



m/e 96 (35)

The CI mass spectrum shows the quasi molecular ion  $(M + H)^+$  to be the most abundant ion.

## 2.5 Melting Range

The melting range of the commercial form of benperidol is 172-174°, determined after drying under vacuum at 60° for 4 hours, using a Gallenkamp electrothermal melting point apparatus with a thermometer range 0-360°. Janssen<sup>8</sup> has reported the melting range of the synthetic product to be 170-171.8°, and that of its hydrochloride salt hydrate to be 134-142°. Benperidol ethanolate has been shown by thermomicroscopic examination<sup>3</sup> to undergo simultaneous melting and desolvation at 140°. Stoichiometry of the crystals was found by thermogravimetric analysis to be benperidol, 1:ethanol, 2. The stoichiometry of the dihydrate was determined in the same way. Hydrated crystals undergo desolvation at 127°, re-crystallising to give a mixture of polymorphic forms I and III.

## 2.6 Differential Scanning Calorimetry

Differential scanning calorimetry (DSC) of the three polymorphic forms of benperidol has been carried out by Azibi et al.<sup>3</sup>, using a Perkin-Elmer, model DSC-1B, calorimeter. Form I was found to produce an endothermic peak at 177° due to melting ( $\Delta H = 41.6 \text{ kJ mole}^{-1}$ ). Form II, obtained by re-crystallisation from isopropanol, melted at 169° ( $\Delta H = 34.9 \text{ kJ mole}^{-1}$ ). Form III, obtained by re-crystallisation from n-heptane, melted at 154°, ( $\Delta H = 29.6 \text{ kJ mole}^{-1}$ ). In the pure state, forms II and III melted without re-crystallising afterwards, but mixtures of forms I and II or I and III melted first and afterwards re-crystallised to give homogenous form I.

In the present work, using a Perkin-Elmer model DSC-2C differential scanning calorimeter, the commercial benperidol sample (Janssen Pharmaceutical Ltd.) melted at 173° (446°K). The form re-crystallised from isopropanol produced a small trough at 153° (426°K), a peak at 169° (442°K),



melting point 166°, a trough at 170° (443°K), and a second peak at 175° (448°K), melting point 173°. There was some discrepancy between the third polymorphic preparation, re-crystallised from n-heptane, and that obtained by Azibi *et al.*, since the former produced a peak at 166° (439°K), melting point 161°. This was followed by a trough at 168° (441°K), and a second peak at 175° (448°K), melting point 172°. According to Azibi *et al.*, the troughs may be due to re-crystallisation of the melted sample to produce form I. The scans obtained are shown in Fig. 9.

## 2.7 Solubilities<sup>8</sup>

Some solubilities of benperidol are given in Table IV. (Data reproduced by kind permission of Janssen Pharmaceutical Ltd., U.K.)

Table IV  
Solubility of Benperidol at Room Temperature

Solvent	Solu- tion pH	Solubility (g per 100 ml)	
water	6.9	about	0.002
0.01 M hydrochloric acid	4.9		0.34
0.1 M hydrochloric acid	1.5		0.19
0.01 M tartaric acid	4.2		0.59
0.1 M tartaric acid	2.5		0.79
0.01 M citric acid	4.2		0.48
0.1 M citric acid	2.6		0.71
3% acetic acid			12.1
6% acetic acid			16
hexane		less than	0.01
chloroform		about	23.2
methanol			0.22
ethanol 95%			0.10
2-propanol			0.60
acetone			0.95
diethyl ether			0.16
ethyl acetate			1.23
benzene			0.59
toluene			0.83

## 2.8 pKa Values

Benperidol has a pKa of 7.90<sup>8</sup>, and is a sufficiently weak base to be extracted by organic solvents from

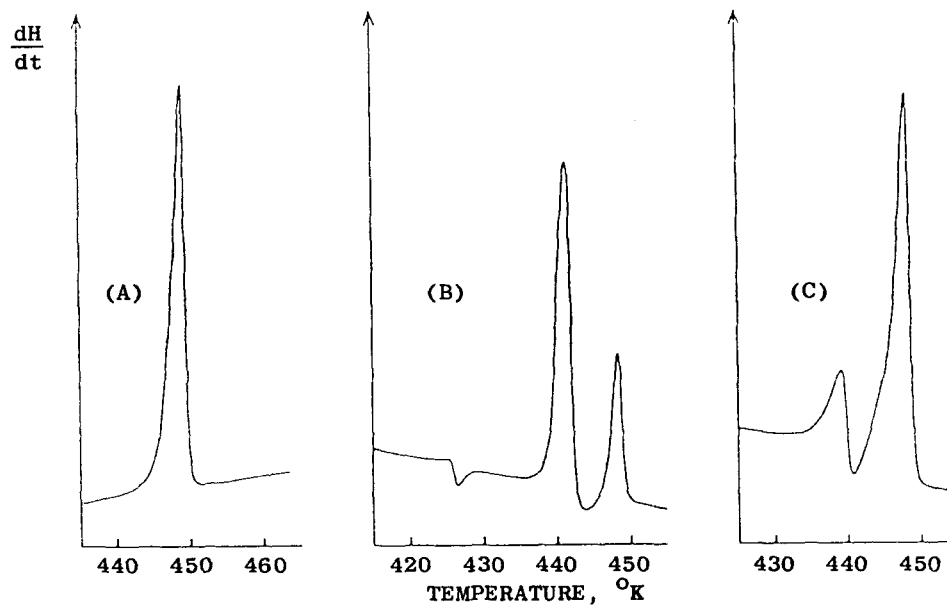


Figure 9. DSC thermograms of benperidol: (A) commercial product; (B) re-crystallised from isopropanol; (C) re-crystallised from n-heptane.

aqueous acid solution<sup>1</sup>. It has been found in the present work that, due to the presence of the benzimidazolone ring system, benperidol can also act as a weak acid when dissolved in strong alkali (pH 11). Evidence of salt formation is seen in the bathochromic shift, from 279 nm to 288 nm, which occurs in the ultraviolet absorption spectrum of benperidol when its aqueous solution is made alkaline (Fig. 10).

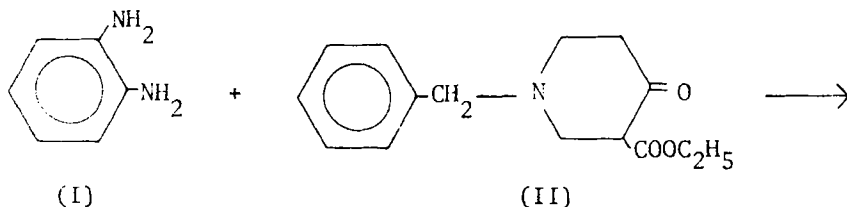
## 2.9 X-ray Diffraction

X-ray diffraction data for crystalline derivatives of butyrophenone drugs have been obtained by Okkerse et al.<sup>9</sup> by the Debye-Scherrer powder technique with photographic recording. Benperidol was crystallised as the free base, picrate, picrolonate, bromhydrate, sulphate, acetate and phosphate.

The free base was re-crystallised from isopropanol, from which it can be assumed to have been polymorph II<sup>3</sup>. The spacings,  $d(\text{\AA})$ , were as follows: 11.0, 9.20, 7.50, 6.95, 6.55, 6.05, 5.90, 5.60, 5.20, 5.05, 4.60, 4.55, 4.20, 4.00, 3.45, 3.35, 2.92, 2.64, 2.10, 1.92. The most intense diffraction lines corresponded to  $d(\text{\AA})$  values of 4.55, 5.20 and 4.00, in order of decreasing intensity.

## 3. Synthesis

The synthesis of benperidol has been patented by Janssen<sup>10</sup>. 1,2-Phenylenediamine (I) is refluxed with 1-benzyl-3-carbethoxy-4-piperidone (II) in xylene to produce 1-(1-benzyl-1,2,3,6-tetrahydro-4-pyridyl)-2-benzimidazolinone (III), which is taken up in acetic acid and alcohol, and hydrogenated at 50° under atmospheric pressure until two equivalents of hydrogen are taken up to give 1-(4-piperidyl)-2-benzimidazolinone (IV). This compound is condensed with 4'-chloro-4-fluorobutyrophenone (V) in 4-methyl-2-pentanone in the presence of sodium carbonate and potassium iodide to give benperidol.



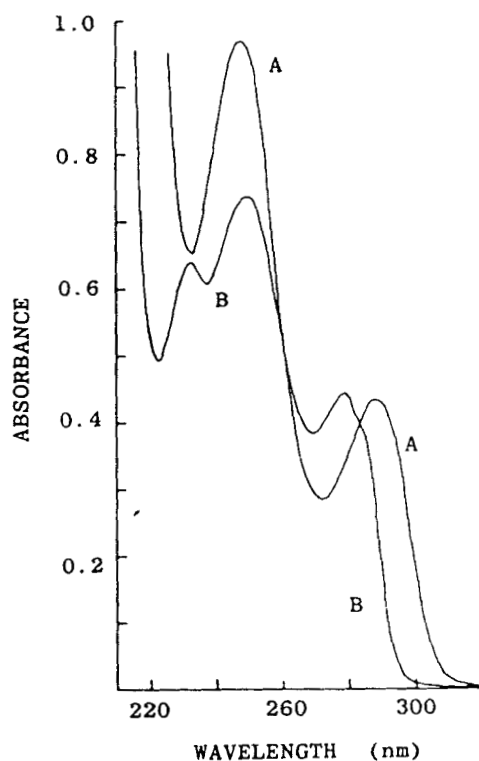
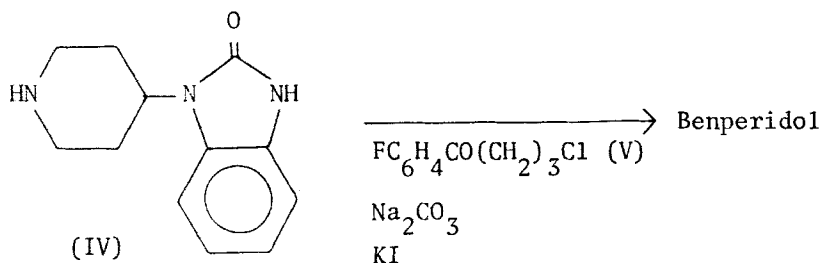
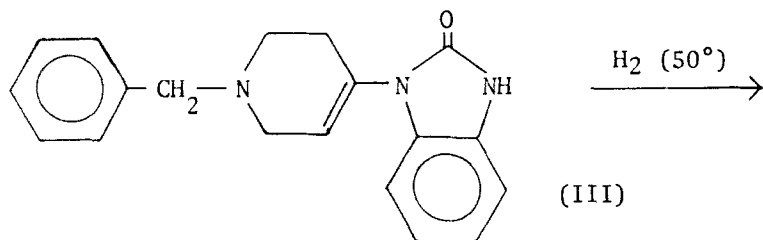
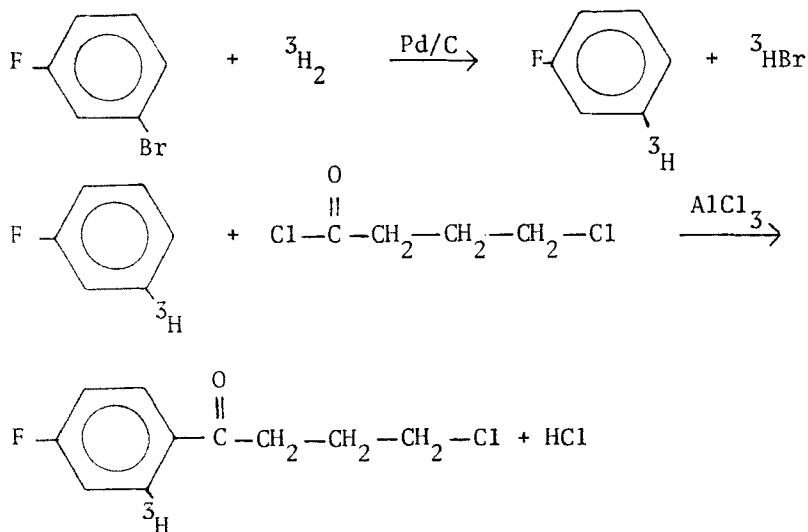


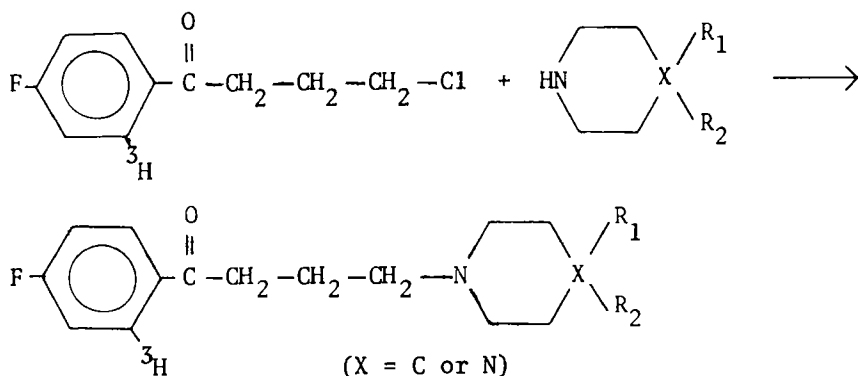
Figure 10. Ultraviolet absorption spectra for  $5.50 \times 10^{-5}$  M benperidol in (A) 0.1M sodium hydroxide, and (B) de-ionized water.



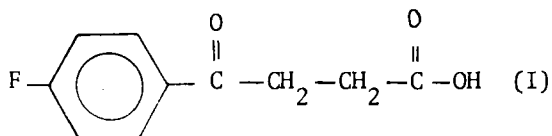
#### 4. Drug Metabolism and Pharmacokinetics

Soudijn *et al.*<sup>16</sup> have studied the metabolism of ten neuroleptic butyrophenone derivatives, including benperidol, after their administration by subcutaneous injection into Wistar rats. All the compounds were labelled by introducing tritium into the fluorophenyl ring as shown in the following reaction scheme.

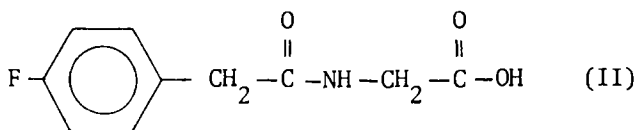




The study was thus limited to the fluorophenyl ring system. Results indicated that oxidative N-dealkylation is a major metabolic pathway for these compounds, and produces  $\beta$ -(p-fluorobenzoyl) propionic acid (I) which is devoid of neuroleptic activity.



This metabolite then undergoes further degradation to p-fluorophenylacetic acid and its glycine conjugate (II) together with a small amount of p-fluorobenzoic acid.



The excretion of radioactive material was maximal within 24 hours. In the case of benperidol, 30.1% appeared in the urine and 54.0% in the faeces within this period. The total recovery of radioactive material obtained from benperidol after 96 hours was 99.8% of which 4.2% was still present in the body.

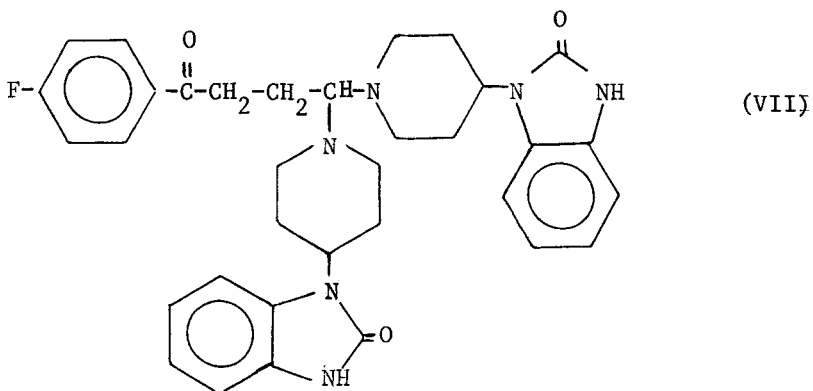
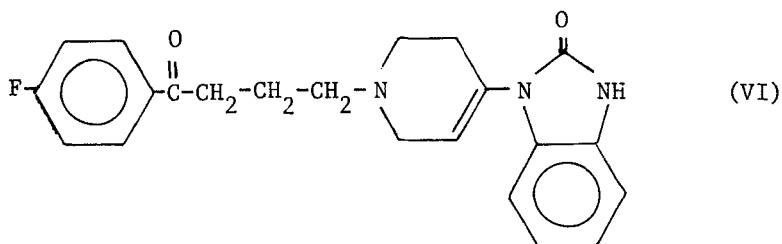
The metabolism of benperidol in man has not been reported, but Forsman et al.<sup>17</sup> have identified p-fluorobenzoyl propionic acid and p-fluorophenylacetic acid as metabolites of haloperidol in man using GLC and mass spectrometry, showing that the metabolic pathway may be the same as in the rat. This study is similarly restricted

however in that the piperidine containing residue of the molecule was not investigated. The findings did suggest <sup>17</sup> however that the apparent absence of psychoactive metabolites may make it possible to establish a relationship between serum levels of butyrophenone drugs and their neuroleptic effects.

### 5. Stability, Degradation and Purity Determination <sup>8</sup>

Benperidol powder is stable, if kept at room temperature (23°C), protected from sunlight and in a closed container. A sample stored under these conditions, but at a higher temperature (about 45°C), showed only a slight colour change from white to a very faint yellow after one year. Exposure to daylight causes a more marked degradation (as much as 7 per cent within one year under normal laboratory conditions), and produces a colour change to yellow or brown. Decomposition can be measured by thin-layer chromatography on silica gel, or alternatively by non-aqueous titration with perchloric acid in glacial acetic acid with  $\alpha$ -naphtholbenzein as indicator, or titration of the fluorine content using thorium nitrate with sodium alizarinesulphonate and methylene blue. The degradation also results in a decrease in ultraviolet absorption, which is most pronounced at the 243 nm maximum, measured in 0.1M hydrochloric acid: isopropanol (1:9). On heating to 100°C in the presence of 1M sodium hydroxide or 1M hydrochloric acid, benperidol gradually decomposes, but in water it remains stable for at least one week. The decomposition in alkali is fairly rapid, and is due to a nucleophilic  $SN^2$  substitution of the fluorine atom by a hydroxyl group, producing 1-[1-[4-(4-hydroxyphenyl)-4-oxobutyl]-4-piperidinyl]-1,3-dihydro-2H-benzimidazol-2-one. Degradation in acid is comparatively slow, and results from a hydrolytic splitting at the piperidine ring nitrogen to give 1,3-dihydro-1-(4-piperidinyl)-2H-benzimidazol-2-one (IV, Section 3). The impurities can be identified by high-performance liquid chromatography using a 30 cm stainless steel column (4.6 mm internal diameter) containing RP R Sil 18 (10  $\mu$ m). An ultraviolet detector is set at 275 nm. Elution is achieved with 0.5% aqueous ammonium acetate solution (eluent A) and a mixture of 70 volumes methanol and 30 volumes of acetonitrile (eluent B). Gradient elution at a flow rate of 2 ml per minute is carried out for 20 minutes until the percentage of eluent B mixed with eluent A is increased from 25% to 75%. The final mixture is then used for isocratic elution. Relative retention times are 0.40 for piperidyl-benzimidazolone 0.74 for the hydroxyphenyl compound and 1.00 for benperidol. The purity of benperidol can be determined by thin-layer chromatography, which allows its separation from 1,3-dihydro

-1-(4-piperidiny1)-2H-benzimidazol-2-one which is designated T358 (IV, Section 3) and 4-chloro-1-(4-fluorophenyl)-1-butanone which is designated T34 (V, Section 3). These compounds are immediate precursors in the synthesis of benperidol. The chromatographic procedure also separates benperidol from two other possible impurities, namely 1-[1-(4-(4-fluorophenyl)-4-oxobutyl)-1,2,3,6-tetrahydro-4-pyridinyl]-1,3-dihydro-2H-benzimidazol-2-one or R4749 (VI) and R35474 (VII)



Pre-coated TLC silica gel F<sub>254</sub> plates (Merck, Darmstadt, F.R.G.), thickness 0.25 mm are used. A quantity (up to 500 µg) of the substance being examined, which may contain up to 100 µg of each of the compounds to be separated, is dissolved in a mixture of 2 volumes of chloroform and 1 volume of methanol and applied to the chromatoplate. The plate is then developed to a height of 15 cm. Spots in the chromatogram are best detected under ultraviolet light (254 nm). The least amounts that can be viewed by this means are 0.1 µg for benperidol and compound R4749, 0.2 µg for compound T34, 0.3 µg for compound R35474 and 0.5 µg for compound T358. Plates are developed using a mixture of ethyl acetate: chloroform: methanol: 0.1M sodium acetate buffer pH 7.4 (54:23:18:5, by volume), or alternatively a mixture of chloroform: methanol: strong ammonia solution



(95:5:1, by volume), solvents I and II respectively. A mixture of chloroform and methanol (90:10, by volume), solvent III, can also be used, but will not separate benperidol and R4749. The  $R_f$  values for T34, benperidol, R4749, R35474 and T358 respectively are 1.00, 0.72, 0.76, 0.54 and 0.08 using solvent I, 0.98, 0.70, 0.64, 0.42, and 0.14 using solvent II, and 0.82, 0.52, 0.52, 0.40 and 0.01 using solvent III.

## 6. Methods of Analysis

### 6.1 Polarography

Benperidol, together with other neuroleptic butyrophenones, has been studied by d.c., a.c. and differential pulse polarography, by constant potential coulometry and by cyclic voltammetry<sup>11,12</sup>. Benperidol, like the other butyrophenones examined, undergoes a two electron reduction resulting in a single irreversible cathode wave that can be used for its determination. Good precision was obtained under alkaline conditions, pH 9 being most suitable for benperidol. The limits of determination by differential pulse polarography were 1 to 2  $\mu$ m.

### 6.2 Fluorimetric Analysis

The fluorescence characteristics of benperidol and some other therapeutically important butyrophenones have been described by Baeyens<sup>13</sup>. Benperidol was found to exhibit strong fluorescence, due to the benzimidazolone fluorophor, with a detection limit of about  $5 \times 10^{-2}$   $\mu$ g ml<sup>-1</sup>. Solutions of benperidol in mixtures of methanol, ethanol or propan-2-ol (2 volumes) with buffer solution (1 volume) produced optimum fluorescence at pH 1-2 and 6-11, but none at pH 3-5. It is suggested that the n-II\* transitions associated with the carbonyl groups of the imidazole structure and of the phenyl-butanone moiety may be responsible for the quenching from pH 3-5<sup>14</sup> and above pH 11<sup>15</sup>. Protonation of the non-bonding electrons may explain the maximum fluorescence at the lower pH.

The intensity of fluorescence and the wavelengths of the excitation and emission maxima depend on the nature of the solvent. Table V shows the variations in the wavelength maxima in some organic solvents. Solutions in methanol are reported to give more intense fluorescence than solutions in higher alcohols. In acetone or chloroform solution, no signal is observed, and the signal in ethyl acetate is weak.

The external heavy-atom effect on the fluorescence of benperidol has been investigated<sup>15</sup> with potassium iodide, thallium (I) acetate, lead tetraacetate and mercury (II) nitrate. A slight increase in the excitation wavelength was accompanied by a decrease in fluorescence intensity, but the emission wavelength was almost unaffected.

Table V

Excitation and Emission Fluorescence Maxima of Benperidol  
(10  $\mu\text{g ml}^{-1}$ ) in Various Solvents

Solvent	Wavelength (nm)	
	$\lambda_{\text{exc}}$	$\lambda_{\text{em}}$
Methanol	235 <sup>†</sup> , 285	320
Ethanol	235 <sup>†</sup> , 288	315
Propan-2-ol	235 <sup>†</sup> , 286	320
Propane-1,2-diol	288	325
Methyl Cellosolve	290	325
Dimethylformamide	290	326
Ethyl acetate	280	315

<sup>†</sup>Weak secondary excitation maxima

### 6.3 Chromatography

TLC procedures, using silica gel 60, have been described by Pluym<sup>18</sup> for the detection and differentiation of psychotropic drugs that are derivatives of butyrophenone, diphenylbutylpiperidine or phenothiazine. Phenothiazine compounds are differentiated from drugs of the other two groups by one-dimensional TLC, with chloroform-ethanol (90:10) as the mobile phase. Benperidol has an  $R_f$  value of 0.55<sup>18</sup> or 0.60<sup>1</sup> using this system. The identification of benperidol (and other butyrophenones or diphenylbutylpiperidines) is confirmed by two-dimensional TLC using ethyl acetate-chloroform-methanol-0.1 M sodium acetate (pH 7.4) (54:23:18:5) as the second mobile phase, and further developing the plate at right angles to the first run.

The British Pharmacopoeia<sup>4</sup> gives a TLC procedure for testing the purity of haloperidol, alone or in tablet or injection formulations. The finding in the present work has

been that the procedure will not serve to separate haloperidol from benperidol or trifluoperidol hydrochloride, respective  $R_f$  values being 0.65, 0.65 and 0.60.

Rüder et al.<sup>19</sup> have determined  $R_f$  values for a number of psychotropic drugs, including benperidol and some other butyrophenones using several azeotropic solvent mixtures as mobile phase, with silica gel plates. Benperidol was separated from trifluoperidol and haloperidol using ethanol:tetrachloromethane (16:84) as mobile phase, the  $R_f$  values being 0.54, 0.63 and 0.74 respectively.

Benperidol can be visualized<sup>18</sup> on a TLC plate by means of iodine vapour or seen under a 254 nm ultraviolet lamp as a dark spot after chromatography on silica gel 60 F254. Alternatively, a spray reagent can be used. Ethanolic solutions of bromocresol purple or bromocresol green, made alkaline with ammonia or sodium hydroxide respectively, will produce light blue spots<sup>18</sup>. Aconite acid in acetic anhydride can be used to form a violet coloured complex<sup>18,20</sup>.

Various modifications<sup>1,18</sup> of Dragendorff's reagent (potassium iodobismuthate solution) will produce an orange-brown spot, as in the British Pharmacopoeia TLC test for haloperidol<sup>4</sup>. Since none of these spray reagents is specific, identification is dependent upon  $R_f$  values, and it is necessary to use a reference sample of benperidol for comparison<sup>18</sup>.

De Croo et al.<sup>21</sup> have studied the effect of various flushing gases (air, nitrogen and helium) on the suppression of oxygen quenching and enhancement of the native fluorescence of some butyrophenone tranquillisers on thin-layer plates. Flushing with nitrogen was found to give enhanced fluorescence with silica gel coated plates, but not with plates coated with cellulose or RP8.

Soep<sup>20</sup> has described a method for the quantitative estimation of chromatographic spots of fluorine containing organic compounds. The procedure, which was studied with haloperidol, depends on the colorimetric determination of the fluoride ion formed after combustion of the fluorine-containing spot in a Schöniger combustion flask.

## 7. Identification and Determination in Pharmaceuticals

Pharmacopoeial methods<sup>4,5,22</sup> are available for the related compound haloperidol, but not for benperidol.

The X-ray diffraction procedures developed by Okkerse et al.<sup>9</sup> (see Section 2.9) can be used for the identification of benperidol and other butyrophenone drugs, either as the free compounds or in pharmaceutical preparations (injections, drops, tablets or coated tablets). Chloroform can normally be used for extraction of the free base from its pharmaceutical formulations. X-ray diffraction patterns have been classified according to "Hanawalt's three strongest lines index" and the "innermost line index" methods, and each drug can be specifically characterised. The method uses larger quantities of the active compound (15 mg in the case of benperidol) than are needed for mass spectrometry<sup>7</sup>.

Haemers and Van den Bossche<sup>23</sup> have developed a general colorimetric procedure for the quantitative determination of butyrophenones in pharmaceutical solutions and tablets. This makes use of the reaction with 3,5-dinitrobenzoic acid in an alkaline medium to give a red coloured complex having a strong absorption maximum at 525 nm. The method has been used for the assay of a sample of benperidol drops which was found as a result to contain 99.3% of the stated content of the drug. The coefficient of variation was 0.9 per cent. Preliminary extraction of preservatives, such as methyl- or propylparaben is unnecessary.

## 8. Identification and Determination in Biological Fluids

### 8.1 Identification

Maurer and Pflieger<sup>24</sup> have described a computerised gas chromatographic-mass spectrometric technique for the identification of benperidol and other butyrophenone and bisfluorophenyl neuroleptics in human urine. The mass fragmentograms obtained were identified using an on-line computer to avoid measuring retention indices. The masses having m/e 112, 123, 134, 148, 169, 257, 321 and 189, 223, 233, 235, 245, 287 and 297 were used for the detection. Urine samples were hydrolysed by refluxing with acid to decompose conjugates of the drug, adjusted to a pH of between 8 and 9, and extracted into an organic solvent mixture (consisting of 2 parts dichloromethane, 2 parts isopropanol, and 6 parts ethyl acetate). Hydroxy- and amino-groups in the extracted compounds were acetylated to improve the GC separation before injection into the gas chromatograph. Benperidol is completely metabolised before excretion in the urine, but its metabolite was detected. Benperidol, droperidol and pimozide, however, each have a common metabolite (m/e 134), while benperidol and pimozide

also show peaks at  $m/e$  259. To distinguish between benperidol plus droperidol or pimozide alone, it was necessary to analyse also an acid extract of the urine. Where benperidol and droperidol have been taken, fluorophenyloxobutanal is detected. Where pimozide has been taken, bisfluorophenylbutyric acid is detected. Androsterone is the only endogenous physiological substance known to appear in the mass fragmentogram.

## 8.2 Chromatography

Marcucci et al.<sup>25</sup> have developed a gas-liquid chromatographic method for quantitatively determining butyrophenones in biological materials. A 2 m glass column was used packed with OV-17 3% on Gas Chrom Q (100-120 mesh). This was operated at 280°. A Ni-63 electron-capture detector was found to be sufficiently sensitive for quantitation in the nanogram range. 2-N-Benzylamino-5-chlorobenzophenone was selected as internal standard, and relative peak area was used as the index of concentration. Mass spectrometry was carried out also in combination with the gas chromatography. Mass spectra obtained in this way for benperidol (and also for fluoropipamide) differed from those obtained after introduction of the sample through a direct inlet. The GLC method was applied only to measuring haloperidol blood levels in patients treated with the drug, and for determining the distribution and metabolism of haloperidol and trifluoperidol in rats. Both of the latter two drugs were found to penetrate very rapidly into the brain, where they reached levels several times higher than in plasma. This confirmed earlier findings<sup>26</sup> made for haloperidol.

Quaglio et al.<sup>27</sup> have described a GLC procedure using OV-1 3% as the stationary phase for the determination of benperidol, droperidol and pimozide in human plasma. Rosenfeld et al.<sup>28</sup> have reported a gas chromatographic method for the analysis of butyrophenones, after derivatization using the Hofmann degradation reaction to give products with shorter retention times. The procedure was applied to haloperidol, droperidol and penfluridol, and the authors suggest that it could be successfully applied to other butyrophenones.

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# TERPIN HYDRATE

Mahmoud M.A. Hassan, Jaber S. Mossa, and  
Abdul Hameed U. Kader Taragan

*King Saud University  
Riyadh, Saudi Arabia*

1. Description	274
1.1 Nomenclature	274
1.2 Formulae	274
1.3 Molecular Weight	277
1.4 Elemental Composition	277
1.5 Appearance, Color, Odor, and Taste	277
1.6 Acidity or Alkalinity	277
2. Physical Properties	277
2.1 Melting Range	277
2.2 Eutectic Temperature	277
2.3 Solubility	277
2.4 Residue on Ignition	278
2.5 Crystallographic Data	278
2.6 Spectral Properties	280
3. Preparation of Terpin Hydrate	290
4. Synthesis of Terpin Hydrate	292
4.1 Total Synthesis	292
4.2 Partial Synthesis	295
5. Biosynthesis of 1,8-Terpin	299
6. Pharmacology	299
6.1 Action and Use	299
6.2 Metabolism	299
6.3 Toxicity	299
6.4 Dosage	302
6.5 Preparations	302
7. Methods of Analysis	302
7.1 Identification	302
7.2 Gravimetric Methods	304
7.3 Titrimetric Methods	305
7.4 Refractometric Method	307
7.5 Determination of Means of Surface Tension	307
7.6 Spectroscopic Methods	308
7.7 Chromatographic Methods	310
References	316



## 1. DESCRIPTION

### 1.1 Nomenclature

#### 1.11 Chemical Names

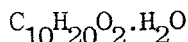
- a) *cis*-p-Menthane-1,8-diol Hydrate (1)
- b) Cyclohexanemethanol, 4-hydroxy- $\alpha,\alpha,4$ -trimethyl monohydrate (2)
- c) p-Menthane-1,8-diol monohydrate (3)
- d) Dipenteneglycol monohydrate (4)

#### 1.12 Generic Names

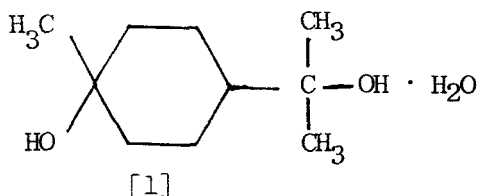
- a) *cis*-Terpin hydrate (4)
- b) Terpin hydrate (5)
- c) Terpinol (2)
- d) Terpinol hydrate (6)
- e) Terpene hydrate (7)
- f) Terpini hydras (7)
- g) Terpinum (2)

### 1.2 Formulae

#### 1.21 Empirical

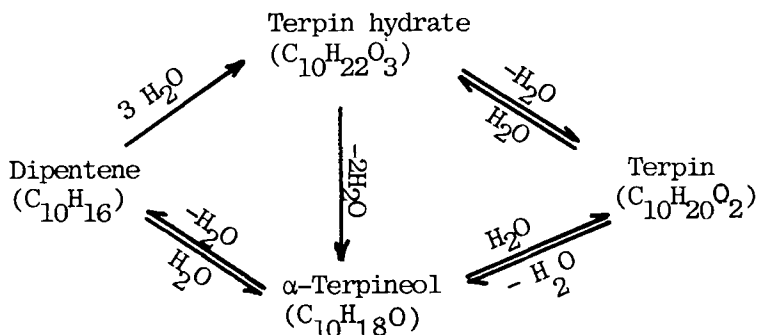


#### 1.22 Structural



Terpin hydrate is a dihydroxy terpin alcohol,  $\text{C}_{10}\text{H}_{20}\text{O}_2 \cdot \text{H}_2\text{O}$ , which is produced by the union of *cis*-terpinol with water. It's relation to dipentene is established by the fact that its dichloride

and dibromide, obtained by the action of the corresponding phosphorus trihalide, are respectively identical with dipentene dihydrochloride and dihydrobromide. Further, dipentene on hydration with 60% sulphuric acid yields terpin hydrate and conversely, terpin hydrate on dehydration yields  $\alpha$ -terpineol and dipentene. The reaction is summarised below :



Terpin hydrate must therefore have the structure [1]. This structure has been confirmed by Perkin and Kay's synthesis ( Scheme 1 ). The structure of terpin hydrate was confirmed also by other authors (8,9). On heating at  $100^\circ$  terpin hydrate loses 10% of its weight which is corresponding to one molecule of water of crystallisation (10,11).

### 1.23 CAS Registry Number

[2451 - 01 - 6]

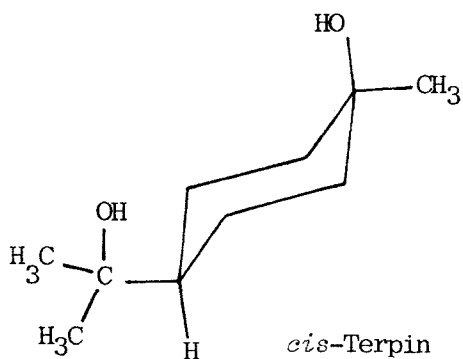
### 1.24 Wiswesser Line Notation

L6 TJ AQ A DX

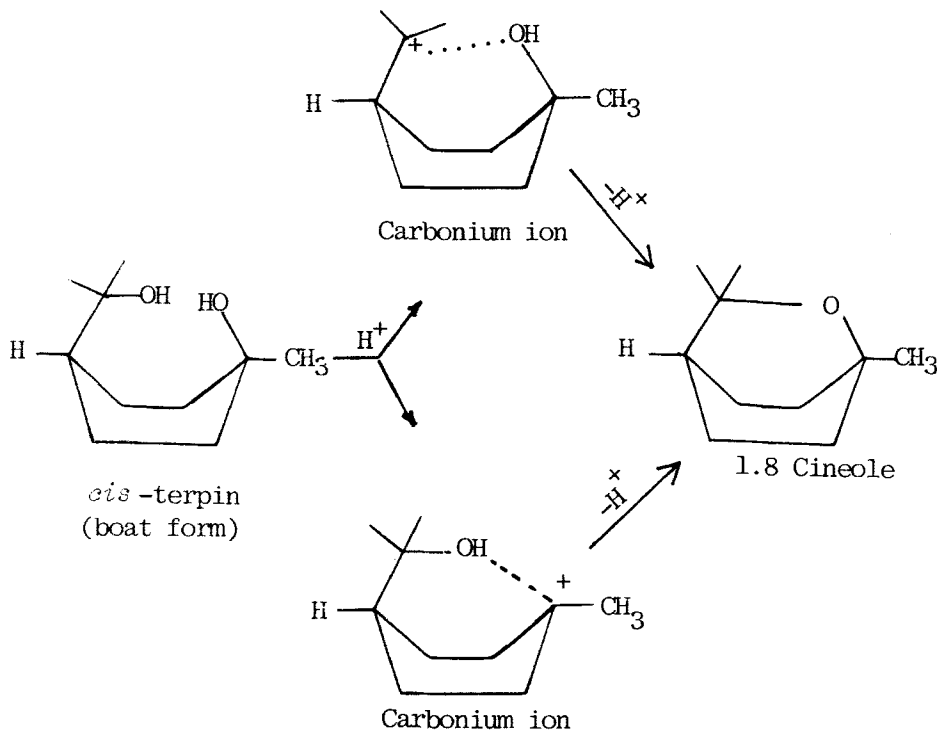
Q & QH C (6).

### 1.25 Stereochemistry

The stereochemistry of 1,8-*cis*-terpin which is also the same for 1,8-*cis*-terpin hydrate is well summarised by Pinder (12). 1,8-*cis*-terpin which melts at  $104-105^\circ$  readily yields a crystalline monohydrate (*cis*-terpin hydrate). Presumably in this form the two hydroxyl groups are in closer proximity, so that a *cis*-configuration is indicated.



The *cis*-terpin is dehydrated by acids to the cyclic ether 1,8-cineol. The formation of the oxide ring will involve the 'boat' conformation of the molecule. *cis*-terpin yields a 'boat' form in which the two hydroxyl groups are in close proximity, and presumably the heterocyclic ring will be formed via either or both possible carbonium ions.



### 1.3 Molecular Weight

190.28 (2)

### 1.4 Elemental Composition

*cis*-Terpin : C = 69.72%  
H = 11.70%  
O = 18.58% (4)

### 1.5 Appearance, Color, odor and taste

Rhombic pyramids from water, colorless lustrous crystals or white powder, efflorescent in dry air; odor: slightly aromatic but not terebinthinate; taste : somewhat bitter (3,4,13).

### 1.6 Acidity or alkalinity

A one percent w/v solution of terpin hydrate in hot water is neutral to litmus (13).

## 2. PHYSICAL PROPERTIES

### 2.1 Melting range

116°-119° (3)

### 2.2 Eutectic temperature

- a) Terpin hydrate-anhydrous terpin : Eutectic at 95° with 10% terpin hydrate (14).
- b) Terpin hydrate-salol : Eutectic at 39° with 97% salol (15).

### 2.3 Solubility

One gram of terpin hydrate dissolves in 128 ml of water, 35 ml of boiling water, 13 ml of alcohol, 140 ml of chloroform, 140 ml of ether, 13 ml of methanol, 13 ml of ethyl acetate, 77 ml of benzene, 290 ml of carbon-tetrachloride and in 250 ml of carbon disulphide. It is also soluble in glycerin and volatile oils. It is insoluble in light petroleum (3-5,16,17).

## 2.4 Residue on ignition

Terpin hydrate yields not more than 0.1 % of residue on ignition (18).

## 2.5 Crystallographic data

Terpin hydrate can be crystallized from water (Fig. 1) Reproducible crystallographic data may be obtainable on anhydrous terpin hydrate produced by sublimation. The crystal structure of terpin hydrate was well summarised by Merone (19).

Crystal Morphology:

Crystal system. Orthorhombic.

Form and habit. Stubby prisms and rods from water showing prism,  $110^\circ$ ; brachy pinacoid  $[010]$ ; and bipyramid  $\{111\}$ . (Fig. 2)

Axial Ratio.  $a:b:c = 0.809:1:0.479$  (x-ray);  $0.8072:1:0.4764$  (1).

Interfacial Angles (Polar).  $110 \wedge 110 = 77^\circ 56'$ ;  
 $101 \wedge 101 = 118^\circ 44'$ ;  $011 \wedge 011 = 128^\circ 48'$ .

X-Ray diffraction data:

Cell dimensions.  $a = 18.51 \text{ \AA}$ ;  $b = 22.87 \text{ \AA}$ ;  $c = 10.96 \text{ \AA}$ .

Formula weights per Cell. 16.

Formula weight. 190.28.

Density. 1.102 (flotation plus pycnometer); 1.098 (x-ray).

### Principal Lines.

d	I/I <sub>i</sub>	d	I/I <sub>i</sub>
8.68	0.57	3.10	0.05
7.17	0.68	2.99	0.08
5.90	0.21	2.91	Very weak
5.69	0.18	2.84	0.18
5.21	0.10	2.78	0.05
4.91	1.00	2.72	0.08
4.69	0.26	2.59	0.31
4.35	0.39	2.43	0.07
3.61	Very weak	2.38	Very weak
3.53	0.16	2.30	0.04
3.44	0.05	2.22	0.04
3.35	0.03	2.17	0.02
3.24	0.08	2.10	Very weak
3.17	Very weak	2.05	0.03

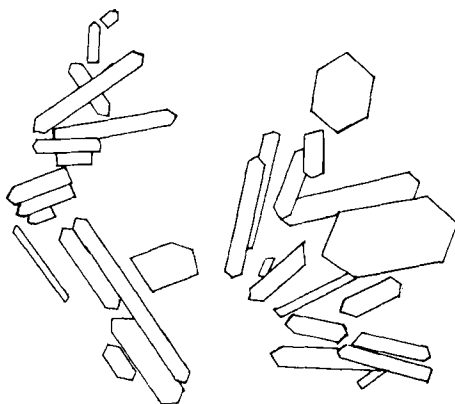


Fig. 1: Terpin hydrate crystals from water.

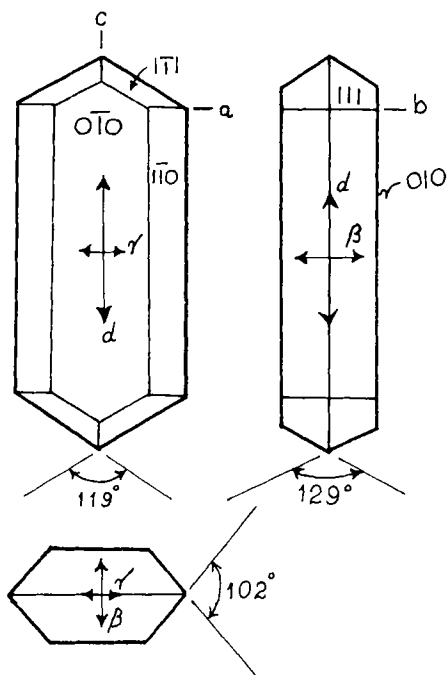


Fig. 2: Orthographic Projection for common habit of Terpin hydrate.

## Optical properties :

Refractive Indexes (5893 Å.; 25 °C)  $\alpha = 1.505 \pm 0.001$ ;  
 $\beta = 1.512 \pm 0.002$ ;  $\gamma = 1.524 \pm 0.001$ .

## Dispersion (I).

	$\alpha$	$\beta$	$\gamma$	2V
Lithium	1.5024	1.5093	1.5211	77° 37'
Sodium	1.5049	1.5124	1.5243	77° 27'
Thallium	1.5073	1.5148	1.5272	77° 18'

Optic Axial Angles (5893 Å.; 25°C.). 2V = 77°; 2E = 143°  
 Dispersion.  $r > r$ .

Optica Axial Plane. 010.

Sign of Double Refraction. Positive.

Acute Bisectrix. a.

Molecular Refraction (R) (5893 Å.; 25°C).  $3\sqrt{\alpha\beta\gamma} = 1.514$   
 $R(\text{calcd.}) = 53.0$   $R(\text{obsd.}) = 52.0$

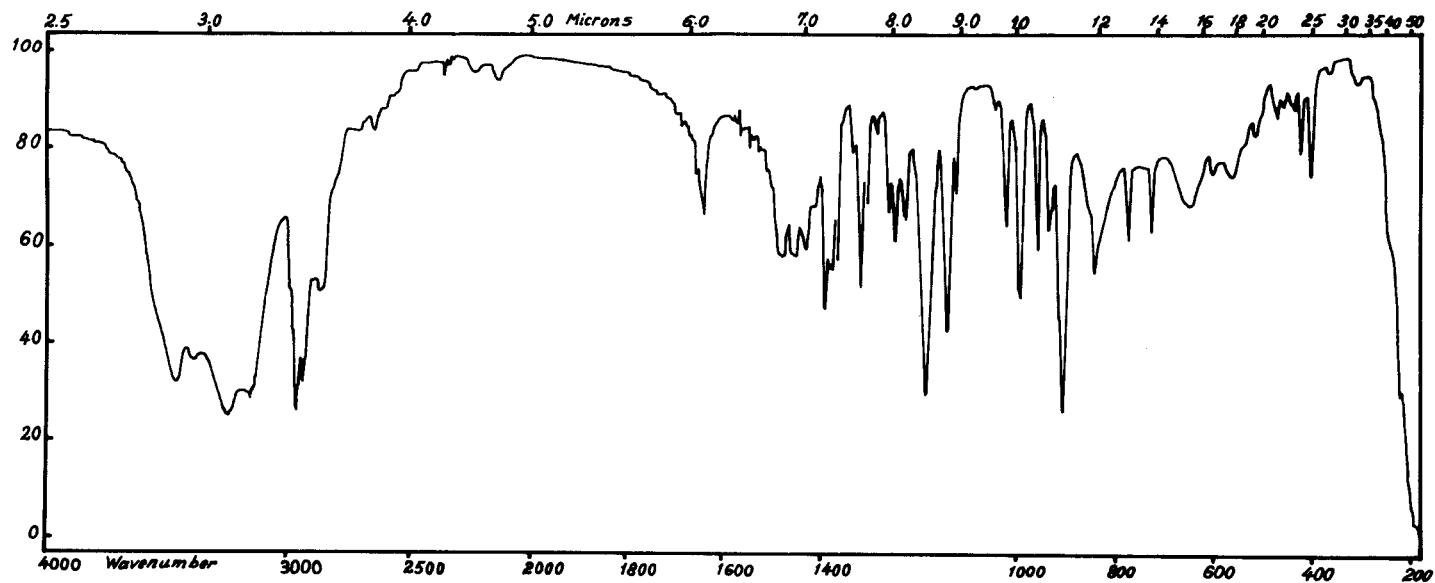
Fusion Data : Terpinol hydrate melts with loss of water indiscriminately between 103° and 120°C. Crystals of the hydrate obtained by recrystallizing from water usually melt at 103° To 105°C.; fusion preparations melt partly at 103° to 105°C., but often other portions of the preparation melt at 115° to 120°C. When melted, the hydrate solidifies spontaneously and slowly as long needles or compact blades, both with moderate birefringence.

2.6 Spectral properties2.61 Ultraviolet Spectrum

The UV spectrum of terpin hydrate in methanol was scanned from 200 to 400 nm using a Pye-Unicam SP 8-100 instrument. No absorbance has been noticed.

2.62 Infrared Spectrum

The IR spectrum of terpin hydrate as KBr-disc is shown in Fig. 3. The KBr-disc was recorded on a Perkin Elmer 580 B infrared spectrometer. The structural assignments have been correlated with the following band frequencies (Table I).



*Fig. 3 IR Spectrum of terpin hydrate as KBr disc*



Table 1. IR Characteristics of terpin hydrate

<u>Frequency <math>\text{cm}^{-1}</math></u>	<u>Group assignment</u>
3470, 3400, 3250	Bonded OH
3160, 2960	CH, $\text{CH}_3$

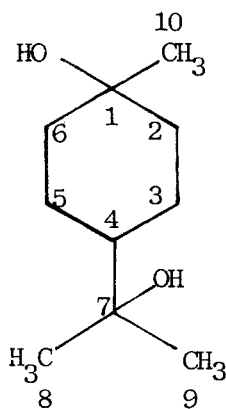
Other characteristic absorption bands are : 1640, 1475, 1450, 1430, 1390, 1360, 1320, 1235, 1220, 1180, 1140, 1020, 990, 960, 940, 910, 840, 775 and  $735 \text{ cm}^{-1}$ . Infrared spectral data were also reported (6).

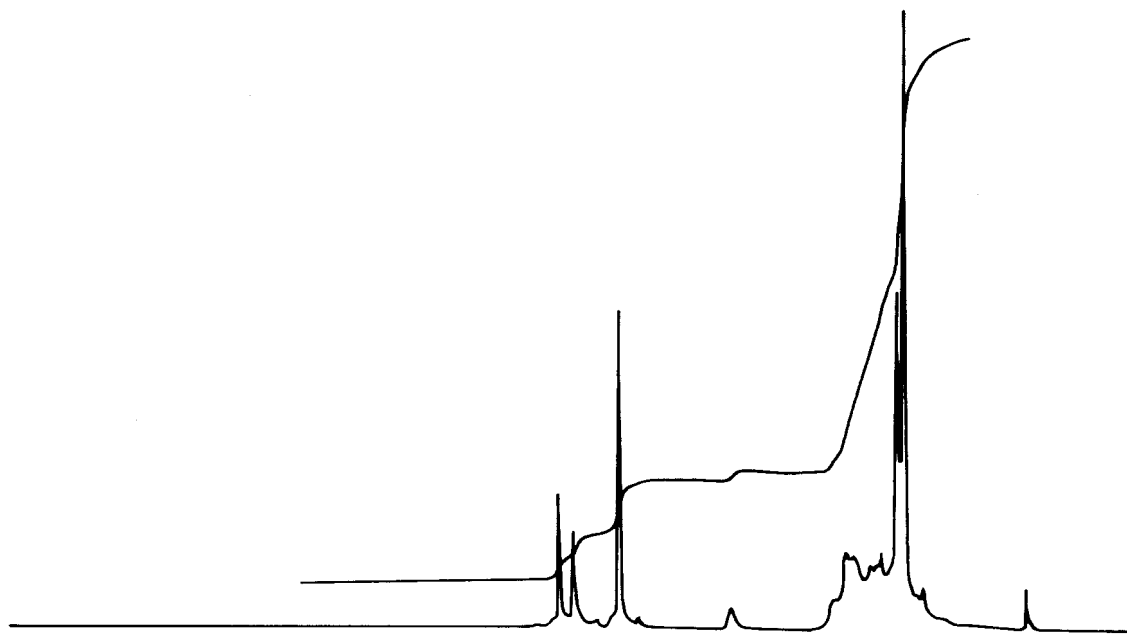
## 2.63 Nuclear Magnetic Resonance Spectra

### 2.631 Proton Spectrum

The PMR spectrum of terpin hydrate in deuterated dimethyl sulfoxide ( $\text{DMSO } D_6$ ) and tetramethylsilane as internal reference was recorded on Jeol-FX-100-90MHz NMR spectrometer (Fig. 4).

The following structural assignments have been made (Table 2).





*Fig. 4 PMR Spectrum of terpin hydrate in DMSO D<sub>6</sub>*

Table 2 : PMR Characteristics of terpin hydrate

<u>Group</u>	<u>Chemical Shift (ppm)</u>
3.97	1 - OH (s)
3.85	7 - OH (s)
3.46	Two H (H <sub>2</sub> O) (s)
1.63-1.08	2,3,5-and 6-CH <sub>2</sub> -protons (m).
1.08	10 - CH <sub>3</sub> (s)
1.04	8- and 9- CH <sub>3</sub> (s).

---

s = singlet.      m = multiplet.

Other reported NMR data are 3.9, 3.8, 3.3, 1.7, 1.1, 1.1, and 1.0 ppm. (6).

#### 2.632 <sup>13</sup>C-NMR spectra

<sup>13</sup>C-NMR completely decoupled and off-resonance spectra of terpin hydrate are presented in Fig. 5 and Fig. 6 respectively. Both were recorded over 5000 Hz range in deuterated dimethyl sulfoxide (DMSO D<sub>6</sub>) (Conc. 100 mg/1 ml DMSO D<sub>6</sub>), on Jeol FX-100-90 MHz instrument. Sample tube 10 mm and tetramethylsilane as internal reference standard at 20°C were used. The carbon chemical shifts are assigned on the basis of the chemical shift theory and the off-resonance splitting pattern (Table 3).

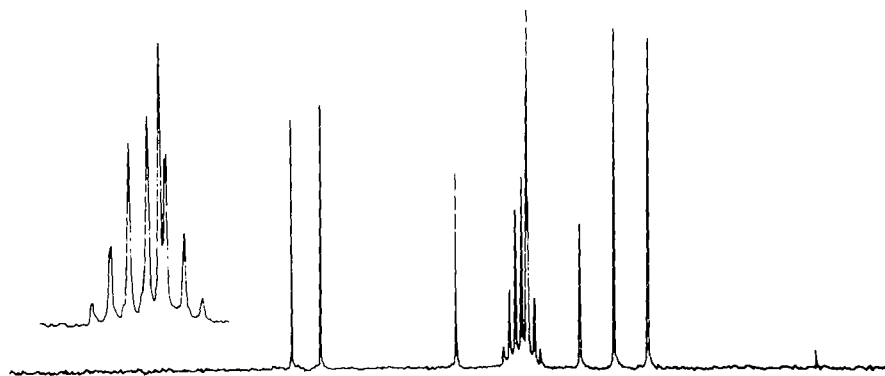


Fig. 5  $^{13}\text{C}$ -NMR completely decoupled spectrum of terpin hydrate in  $\text{DMSO}-d_6$

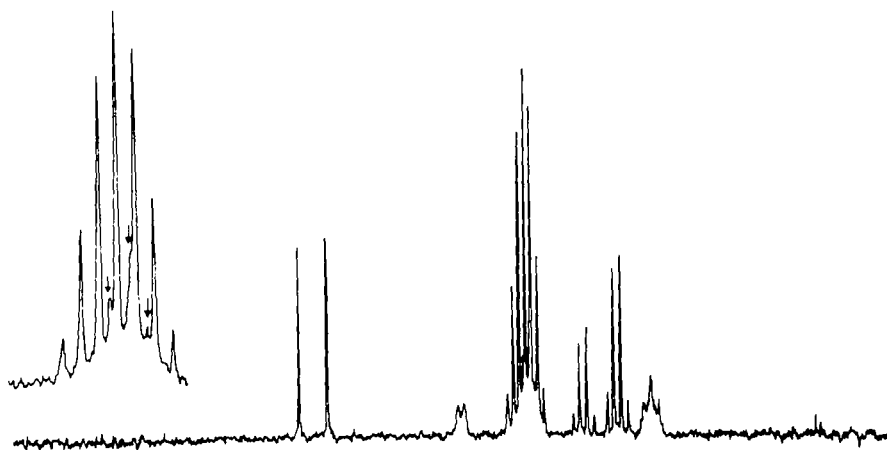


Fig. 6  $^{13}\text{C}$ -NMR off-Resonance spectrum of terpin hydrate in  $\text{DMSO}-d_6$

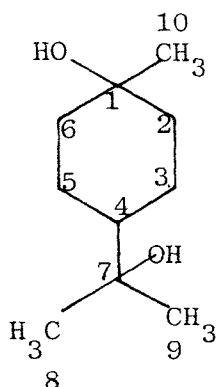


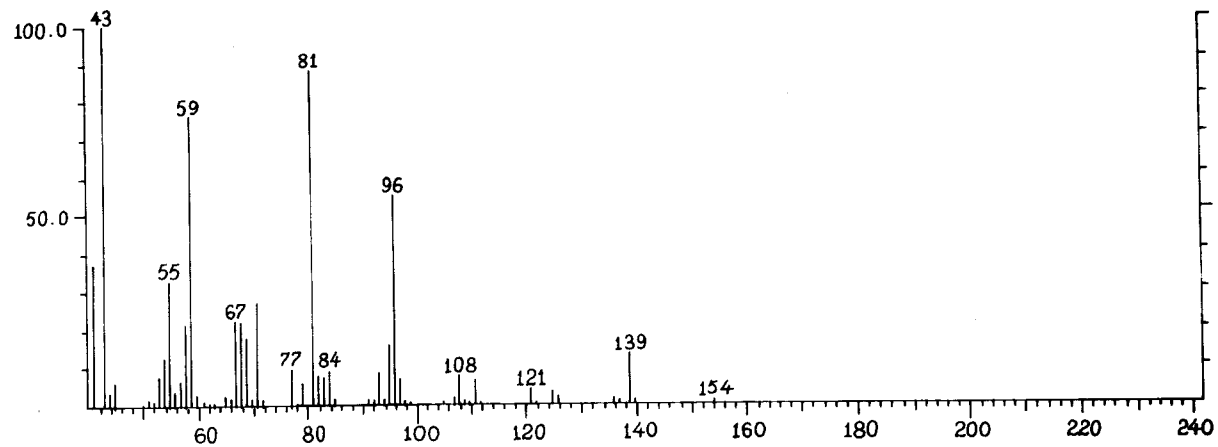
Table 3 : Carbon chemical shifts of terpin hydrate

Carbon No.	Chemical shift (ppm)	Carbon No.	Chemical shift. (ppm)
C - 1	70.86(s)	C - 6	38.63 (t)
C - 2	38.63(t)	C - 7	66.87 (s)
C - 3	22.37(t)	C - 8	26.83 (q)
C - 4	48.32(d)	C - 9	26.83 (q)
C - 5	22.37(t)	C - 10	31.47 (q)

s = singlet; d = doublet; t = triplet  
q = quartet.

## 2.64 Mass spectrum

The mass spectrum of terpin hydrate obtained by electron impact ionization at 70 eV, was recorded on a Finigan-Mat 1020 mass spectrometer. The spectrum (Fig. 7) shows a molecular ion peak  $M^+$  -18 at  $m/e$  154. The base peak is at  $m/e$  43. The most prominent fragments and their relative intensities are shown in Table 4.



*Fig. 7 (EI) Mass spectrum of terpin hydrate*

Table 4: Mass Fragments of Terpin Hydrate

<u>m/e</u>	<u>Relative intensity</u>	<u>Fragments</u>
154	1.08	$M^+ - 18$
		<p style="text-align: center;"> <chem>CC1(C)CCCCC1O</chem> <math>\xrightarrow{-H_2O}</math> <chem>CC1=CCCCC1</chem> or <chem>CC1(C)C=CC(O)CC1</chem>  172.77 (154.27) </p>
139	12.82	<p style="text-align: center;"> <math>\left[ \text{Cyclohex-2-en-1-ol-1,1-dimethyl} \right]^+</math> or <math>\left[ \text{Cyclohex-2-en-1-ol-3,3-dimethyl} \right]^+</math> </p>
121	3.91	<p style="text-align: center;"> <math>\left[ \text{Cyclohex-2-en-1-yl-1,1-dimethyl} \right]^+</math> </p>
108	7.29	<p style="text-align: center;"> <math>\left[ \text{Cyclohex-2-en-1-yl-3,3-dimethyl} \right]^+</math> </p>
96	56.1	<p style="text-align: center;"> <math>\left[ \text{Cyclohex-2-en-2-yl-1,1-dimethyl} \right]^+</math> </p>

81	87.62	$\left[ \text{C}_6\text{H}_{10} \right]^{\dagger}$
69	17.26	$\left[ \text{C}_6\text{H}_9^+ \right]^{\dagger}$
68	21.52	$\left[ \text{C}_6\text{H}_9 \right]^{\dagger}$
55	32.76	$\left[ \text{C}_6\text{H}_9^+ \right]^{\dagger}$
43	100.00 (base peak)	$\left[ \text{CH}_3 - \text{CH}^+ - \text{CH}_3 \right]^{\dagger}$



### 3. PREPARATION OF TERPIN HYDRATE

Terpin hydrate is of considerable importance, technically being used as an intermediate in perfumery for the manufacture of terpineol and in pharmaceutical industry due to its disinfectant qualities, as an expectorant. It has also been suggested as a means of improving the durability of papers, cardboard and cartons. It may also be used to fix essential oils. Occurrence of terpin hydrate in nature is rare (20, 21). Terpin hydrate is usually prepared from oil of turpentine (Route VII). It is also obtained from red and white camphor oils (22-25). A tremendous amount of work was done on the production of terpin hydrate from oil of turpentine (26-46). The commercial production of terpin hydrate is well summarised below (47,48):

#### i) Using sulphuric acid:

##### a) Direct agitation process:

A lead-lined pine wood vat with a height of 3 meters is filled to a height of 1.9 meters with sulphuric acid ( $60^{\circ}$ ) which is diluted with water to the concentration of 14%. Then 600 kg of oil of turpentine are charged in, equivalent to a height of 40 cm. The oil-acid mixture is then agitated continuously with a stirrer and the temperature is maintained at about  $25-30^{\circ}$ . During this agitation the contents of the vat become homogeneous and form a turbid yellowish mass. Heat is generated by this reaction and cooling is necessary to keep the temperature around  $30^{\circ}$ . The formation of terpin hydrate is followed from the rise in density of the liquid. The stirring is continued uninterruptedly until the density is 0.905-0.910. When the required density has been reached, the spend oils are drawn off and the acid liquid is siphoned off. In the course of manufacture a part of terpin hydrate formed during the agitation is extracted from time to time. This is centrifuged in a lead-lined apparatus. The remaining terpin hydrate lining the vat are removed with wooden hammers and are transferred to the crusher. The crude terpin hydrate thus obtained is charged in a lead lined kettle containing boiling water and then stirred for 15 minutes. The terpin hydrate solution is then drained into a crystallising vessel and allowed to crystallise. If necessary it is treated with magnesium carbonate and animal charcoal,

filtered and crystallised. Finally the terpin hydrate is centrifuged and dried.

b) Contact process:

A mixture of 420 kg pure oil of turpentine and 200 kg pine wood sawdust with exclusion of air is stirred thoroughly in 1500-1800 litres lead-lined kettle for few hours. The slurry is left for several hours to permit the complete absorption. 200 kg of commercial sulphuric acid (60°) with a strength of 25% is prepared with enough water and then stirred into the turpentine slurry vigorously. A wooden cover is placed over the kettle. The reaction mass is then left for 10-14 days. The success of the operation depends solely on intimate contact between the oil of turpentine with its relatively low density and dilute sulphuric acid of higher density. The contact is effectively promoted by pine wood sawdust. After 10-14 days, the mass in the kettle will swollen and become transformed into a cake permeated with crystals of terpin hydrate. The mass is crushed with a spade. The excess of acid and the unreacted oils are run off. The contents of the kettle are washed several times with pure water and then finally with water containing a little carbonate.

The sawdust is charged into a distilling apparatus and steam-distillation is carried out. Oil of turpentine comes over first. When all the oil has been entrained the distillation is stopped and the residue is heated at 100° with 400 litres of water. This heating causes the terpin hydrate to be dissolved and to be extracted from sawdust. The terpin hydrate solution is then separated from the sawdust. The terpin hydrate which is crystallised from the water on cooling is sucked to dry or centrifuged.

ii) Using nitric acid:

A mixture of 150 litres of oil of turpentine and 100 litres of nitric acid (17°-21°) is stirred continuously into 300 litre stone ware or aluminium vat. The mixture is kept in movement with a wooden stirrer for several days. Continuous night and day working is recommended for better yield. If they are left motionless hot spots are developed which spread rapidly and spoil the process. Each day at short intervals samples are taken from each vat to check the extent of terpin

hydrate formation. When the process is complete, the contents of the vats are poured through aluminium screen and the crude terpin hydrate retained on the screen is freed from liquid by centrifugation.

iii) Using sulfuric and nitric acids:

Terpin hydrate is also prepared commercially by treating turpentine oil with sulphuric acid, nitric acid and alcohol. The mixture is agitated continuously for 4 to 6 days. On standing for several days the crude terpin hydrate is crystallized out which is then purified by recrystallization from alcohol.

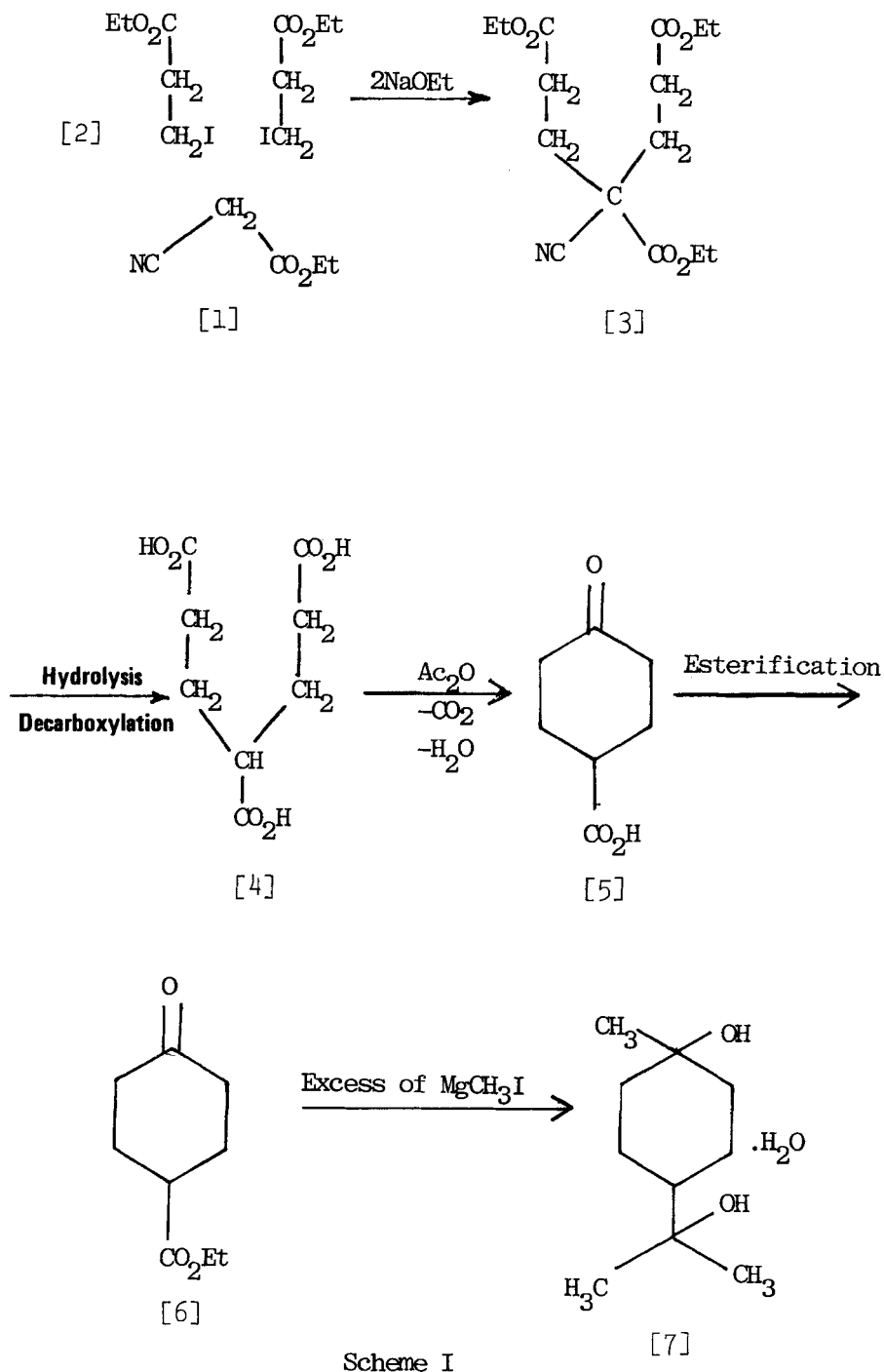
#### 4. SYNTHESIS OF TERPIN HYDRATE

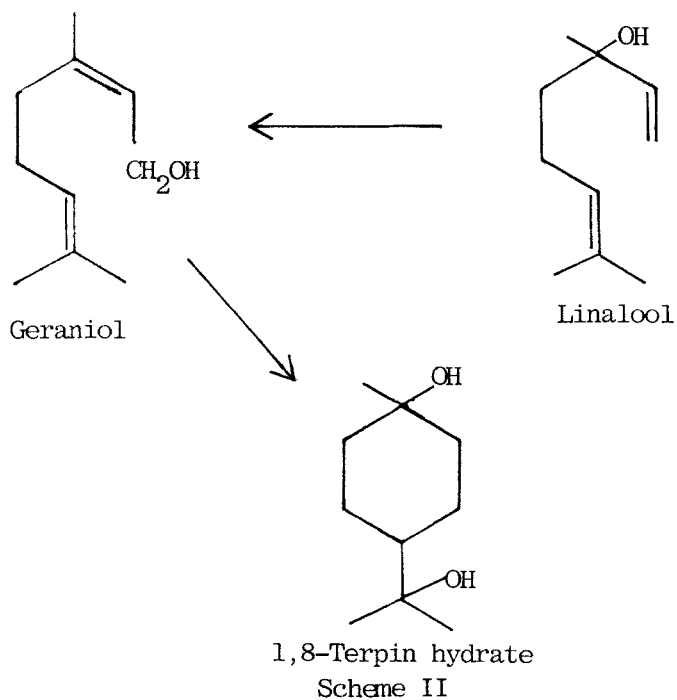
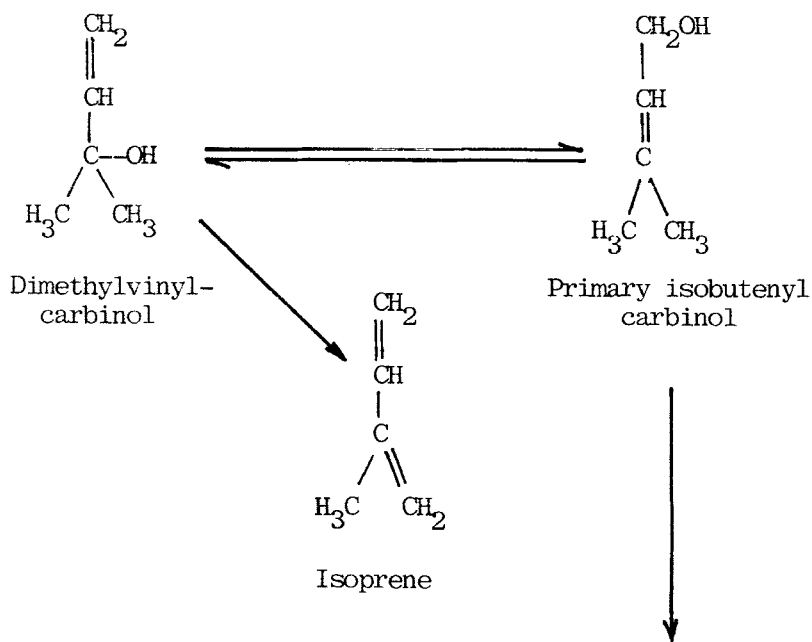
##### 4.1 Total synthesis

Perkin (49,50) who had devised a complete synthesis of terpineol, also found a synthetic route to 1,8-terpin hydrate (Scheme I). This can be achieved by condensation of ethyl cyanoacetate [1] with two molecules of ethyl-  $\beta$ -iodopropionate [2]. The cyanoester [3] obtained is hydrolysed and decarboxylated to the tricarboxylic acid (  $\gamma$ -carboxy pimelic acid [4]. The reactions of which with acetic anhydride followed by distillation gave cyclohexanone-4-carboxylic acid [5]. The ethyl ester [6] of which gave 1,8-terpin hydrate [7] by reaction with methyl magnesium iodide.

Another interesting total synthesis (Scheme II) for the synthesis of terpin hydrate has been disclosed by Favorsky and Lebedera (51) and is as follows:

Acetylene is condensed with acetone in presence of potassium hydroxide yielding an acetylenic alcohol which is then electrolytically reduced in alkaline solution to dimethylvinylcarbinol. The latter is dissolved in 20 times the quantity of 20% sulphuric acid allowed to stand 4-5 days, and the resultant two layers are separated. One of these is water-insoluble and contains isoprene while the other is water-soluble. On neutralisation and fractionation of the latter, a hydration product of dimethylvinylcarbinol is obtained as the second fraction in the form of  $\alpha,\alpha$ -dimethyltrimethylene glycol while from the third fraction terpin hydrate (m.p. 114-116°C) is obtained.



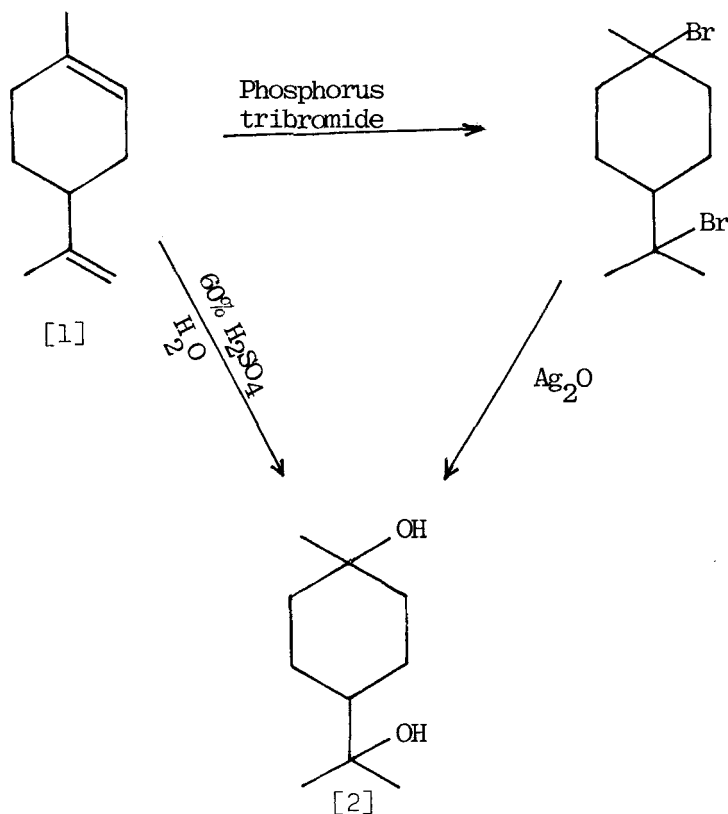


## 4.2 Partial synthesis

Seven routes for the partial synthesis of terpin hydrate are described:

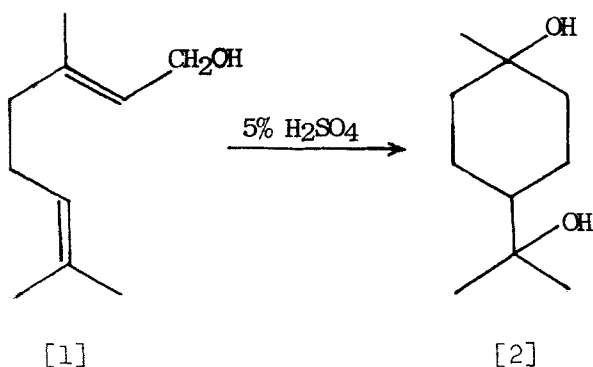
### Route I

Limonene or dipentene [1] is converted into terpin hydrate [2] by direct addition of water at the two double bonds or through intermediate addition of hydrogen halide followed by hydrolysis with silver oxide (47,52-54).

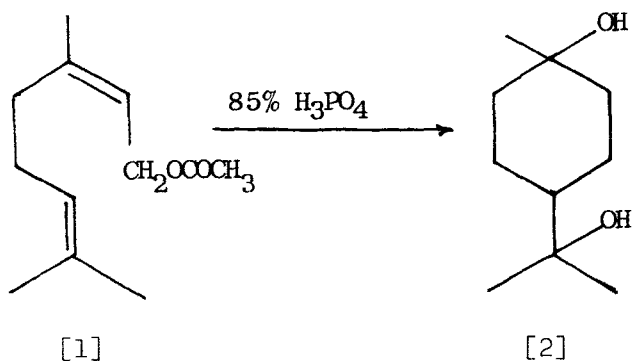


Route II From geraniol and nerol

Geraniol or nerol [1] undergoes rearrangement to terpin hydrate [2] merely on shaking with 5% dilute sulphuric acid (55).

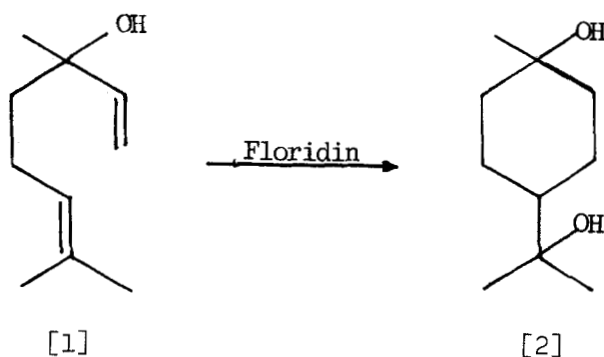
Route III From geranyl acetate

Isagulyants and Serebrennikov (56) effected the isomerisation of geranyl acetate [1] into terpin hydrate [2] by treatment with 85% phosphoric acid.

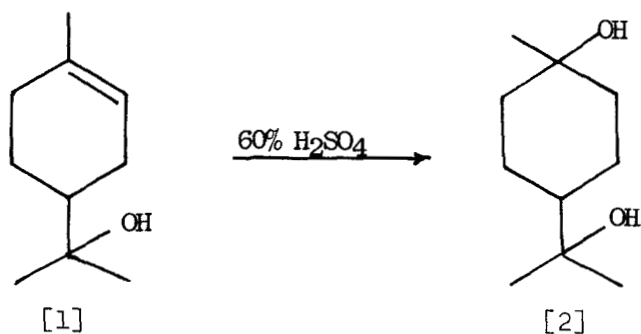


Route IV From Linalool

Linalool [1] is isomerised with Hcl-activated slicate (floridin) into terpin hydrate [2] (57).

Route V From  $\alpha$ -terpineol

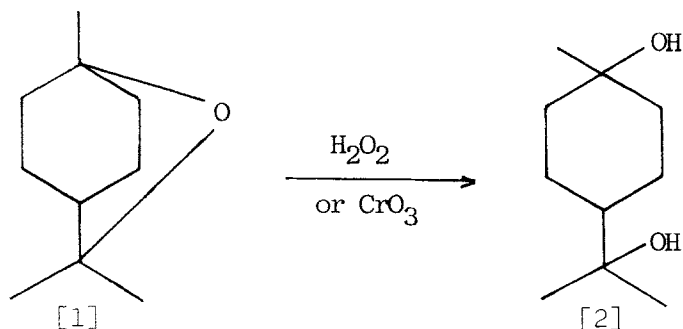
$\alpha$ -terpineol [1] when treated with dilute sulphuric acid is hydrolysed to terpin hydrate [2] (58).



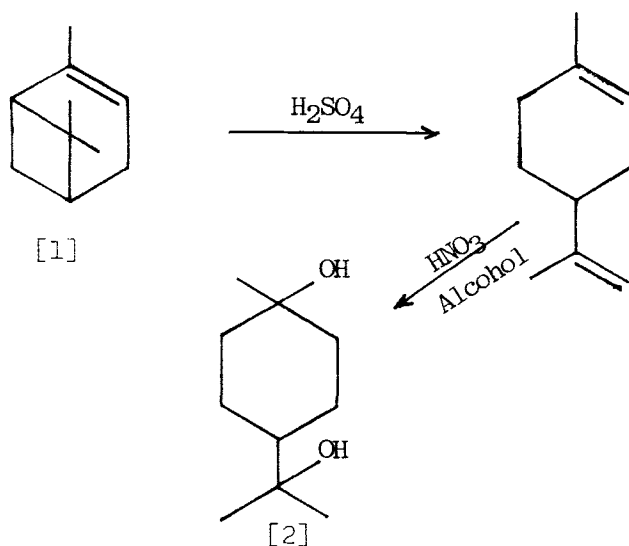


Route VI From cineol

Cineol [1] on oxidation with hydrogen peroxide or chromic acid yield terpin hydrate [2] (59,60).

Route VII From  $\alpha$ -pinene

Hydration of  $\alpha$ -pinene [1] with sulphuric acid hydrochloric acid or nitric acid with or without the presence of alcohol yield terpin hydrate [2] (61-68). The reaction involves a rupture of the 1,6-menthano bridge of the pinene with simultaneous rearrangement and addition of sulphuric acid to the double bond. Upon hydrolysis of the latter, the two molecules of sulphuric acid are rearranged and the glycol terpin (*cis*-p-menthane-1,8-diol) is formed. On standing terpin forms the hydrate [2].



## 5. BIOSYNTHESIS OF 1,8-TERPIN

Ruzicka (69,70) proposed a unified biogenetic scheme for terpenes. The biosynthetic building blocks for these terpenes are isoprene units. The principal route from acetate through mevalonate to the biosynthetically active isoprene units such as isopentenyl pyrophosphate [1] and dimethyl allyl pyrophosphate [2] is described in Scheme III. Geranyl pyrophosphate [3] is the C-10 precursor for the terpenes (10). The biosynthesis of 1,8-terpin is well summarised by Goodwin and Merecer (71) Scheme IV.

The isopentenyl pyrophosphate [1] derived from mevalonic acid isomerized into dimethyl allyl pyrophosphate [2] and the subsequent condensation yield geranyl pyrophosphate [3]. The geranyl pyrophosphate again is isomerized into neryl pyrophosphate which is also formed by isomerization of linaloyl pyrophosphate [5]. The neryl pyrophosphate will be converted into a common enzyme bound cyclic intermediate  $\alpha$ -terpineyl cation [6] which undergoes a hydride shift to form the ion [7], related to terpin-4-ol. Further hydroxylation of [7] gives  $\alpha$ -terpineol [8] and then finally 1,8-terpin [9].

## 6. PHARMACOLOGY

### 6.1 Action and use

Terpin hydrate has been stated to increase the bronchial secretion and to assist expectoration. It is used in chronic bronchitis and in the treatment of unproductive cough (3, 72-74). An alkali-treated terpin hydrate, heated with pretreated maltose and mixed with rosin is an effective non-toxic antidote to nicotineism and has prophylactic properties when mixed with cigarette tobacco (75).

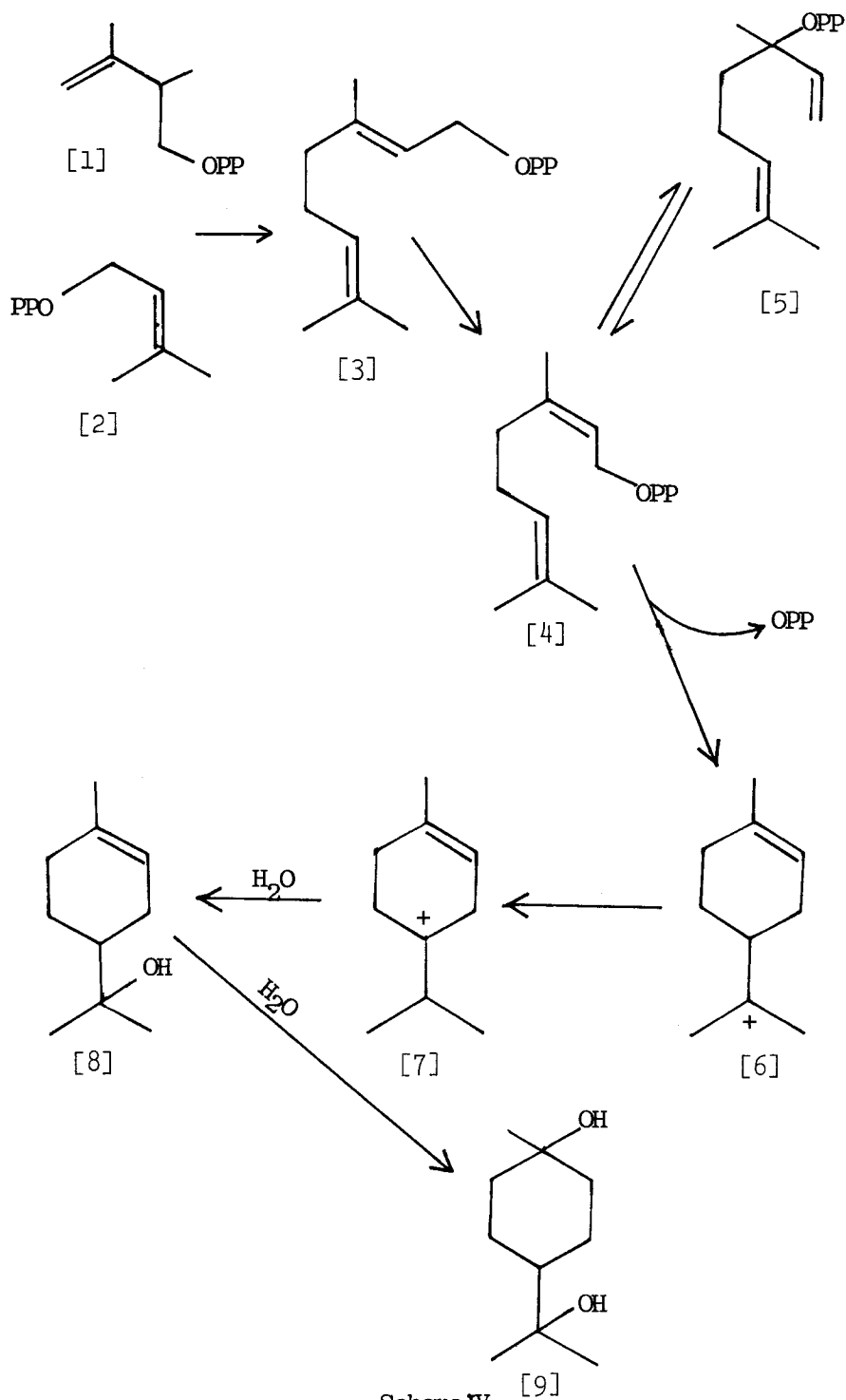
### 6.2 Metabolism

Evidences to support the belief that in the body terpin hydrate is slowly converted into therapeutically active terpenes is lacking (76).

### 6.3 Toxicity

Epigastric pain may follow the administration of terpin hydrate on an empty stomach (77). It will also produce nausea and vomiting (74).





#### 6.4 Dosage

300–600 mg. every 6 hours (3, 78).

#### 6.5 Preparations

The following are the preparations of terpin hydrate official in National Formulary (18).

- a) Terpin Hydrate Elixir
- b) Terpin Hydrate and Codeine Elixir
- c) Terpin Hydrate and Dextromethorphan Hydrobromide Elixir

### 7. METHODS OF ANALYSIS

#### 7.1 Identification

##### 7.11 Pharmacopeial tests

The following tests have been described in Pharmacopoeia, for the identification of terpin hydrate:

- i) Heat at 100°C; terpin is formed and sublimes in fine needles.
- ii) Dissolve 0.1g in 5 ml of hot water and add 0.2 ml of sulphuric acid, the liquid becomes turbid and acquires an odor of lilac, due to the formation of terpineol (7).

##### 7.12 Other tests

###### a) Iodine test

Terpin hydrate reacts with iodine, on warming, to form a liquid compound which is heavier than water and gives no reaction with starch. The liquid is readily decomposed with the evolution of large quantities of iodine and hydrogen iodide, if exposed to light and air for few hours (79).

###### b) Color tests

The following color tests have been described for identification of terpin hydrate:

- i) When 0.05 g of terpin hydrate is treated with 2 ml of concentrated sulphuric acid, an orange-red colour is produced (80).
- ii) With vanillin sulphate or vanillin-hydrochloride solution, terpin hydrate remains yellow, turns green on heating and then dark blue on cooling (80,81).
- iii) To about 2 ml of a 0.05% alcoholic solution of terpin hydrate add 5 ml of 5% phosphomolybdic acid solution and 5 ml of sulphuric acid. A dark green precipitate is formed which disappears on shaking; the green liquid rapidly turns dark blue (80).
- iv) With benzene and sulphuric acid, terpin hydrate gives orange-yellow ring (80).
- v) With furfural and sulphuric acid terpin hydrate gives intense red color. In the modification of the test by adding sulphuric acid without agitation terpin hydrate gives reddish-yellow ring (upper-zone turning purple) (80).
- vi) A small amount of terpin hydrate is treated with 5-10 drops of 1% solution of p-dimethylamino-benzaldehyde in dilute ethyl alcohol. To this solution of the substance and the reagent, add carefully sufficient quantity of sulphuric acid (12%). A blood red ring is formed (82).
- vii) Moisten the terpin hydrate with ethyl alcohol (85%) and allow the alcohol to evaporate, characteristic crystals are formed. If a drop of sulphuric acid is added to the latter, a yellow coloration will develop which will change to orange on heating. Finally a carmin-red color will develop on the addition of a drop of solution containing about 0.5 g of fructose in 25 ml of ethyl alcohol (90-95%) and 7 ml of water (83).
- viii) When terpin hydrate is treated with excess of Deniges' reagent, an amorphous yellow precipitate is produced (84).

c) Microcrystal test

Nikolics (85) has reported a microcrystal test for the identification of terpin hydrate. About 0.02 g

of terpin hydrate is dissolved in a solvent mixture containing 3 ml of ethyl alcohol and 0.04 ml ethyl acetate. The solution is allowed to evaporate gradually on 25 mm. object carrier disc. Surface cralling crystals with dentate edges will arise from one nucleus (Fig. 8).

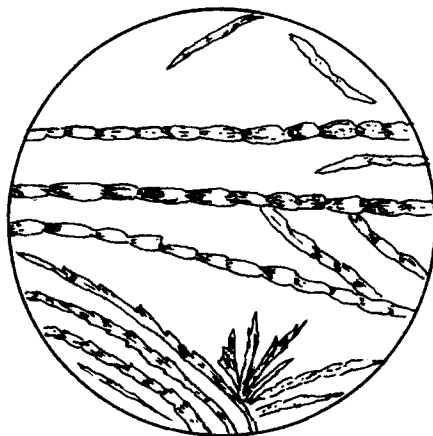


Fig. 8: Terpin hydrate crystals form ethanol ethyl acetate mixture.

## 7.2 Gravimetric Methods

Murray has developed a suitable gravimetric assay for the estimation of terpin hydrate in elixirs (86-88). The procedure used is as follows:

Procedure: Extract a measured quantity of the elixir of the sample with petroleum ether to remove volatile oils. Then dilute the elixir with saturated sodium chloride solution until the ethanol content is reduced to about 10 to 15%. Shake out with four portions, one-fourth volume each, of chloroform containing 5-7 per cent ethanol by volume. Wash each portion of the solvent successively with 5 ml. of the prepared sodium chloride solution. Filter through a pledget of purified cotton into a tared beaker or small crystallizing dish, finally rinsing the cotton and the tip of the funnel with a

little alcohol. Evaporate with the aid of a blast of air without the application of heat. Wipe off any moisture that may have collected on the outside of the dish and allow to stand fifteen minutes before weighing. Samples containing no volatile oils can be assayed by the method, without the preliminary treatment with petroleum ether.

Carol (89) proposed a gravimetric method for the determination of terpinol hydrate in elixir of terpinol hydrate and codeine. The method is as follows:

Measure 10 ml of the sample into a separator. Add 10 ml of water and 5 ml of 10% sulphuric acid and immediately extract with 25 ml of petroleum ether. Drain the aqueous layer into a second separator. Wash petroleum ether twice with 2 ml portions of water and add washings to the aqueous layer. Completely extract the aqueous solution with chloroform-alcohol (95+5). Combine chloroform-alcohol extracts and wash with 5 ml of 7-8% sulphuric acid. Filter the extract into a tared dish. Evaporate in a slow current of air to constant weight. Weigh the residue and report as g terpin hydrate/100 ml.

The above described method was modified in two ways :  
a- taking separate portions of elixir for the determination of terpin hydrate and codeine respectively;  
b- providing spontaneous evaporation of terpin hydrate extract in a current of air dried by means of a sulphuric acid train. Collaborative study of the modified method gave better results and its adoption as tentative is recommended (90). This modified technique is described in detail in "Official Method of Analysis" (91).

### 7.3 Titrimetric Methods

#### 7.3.1 Aqueous

Bostoganashvili (92) has described a volumetric method for the determination of terpin hydrate in commercial terpin hydrate or tablets. The method is as follows:

Add 5 to 10 ml of 10% sulphuric acid to 0.3 g of sample (commercial terpin hydrate, or tablets), pass steam into the mixture for 15 min., then heat the flask, and steam-distil for 15 min., collecting the resulting terpineol in 10 to 15 ml



of ethanol. To the absorbent solution, add 35 ml of 0.1 N potassium bromate solution, 10 ml of 10% potassium bromide solution and 10 ml of dilute sulphuric acid. Set aside in the dark for 15 min., add 10 ml of 10% potassium iodide solution, set aside in the dark for 5 min., and titrate with 0.1N sodium thiosulphate in the presence of starch.

An indirect vanadometric method of assay for terpin hydrate was proposed by Gurevich and Goltman (93). The method involves the oxidation of terpin hydrate by ammonium vanadate. Terpin hydrate is oxidised by ammonium vanadate in a medium of sulphuric acid, the reaction being carried out in a special flask; unconsumed ammonium vanadate is titrated with standard ammonium ferrosulphate solution. From 1.5 to 15 mg of terpin hydrate can be determined with an error of  $\pm 0.8\%$ .

#### 7.32 Non-aqueous

A non-aqueous titration method is described for the analysis of terpin hydrate (94). The method involves the reaction of 3,5-dinitrobenzoyl chloride with terpin hydrate in pyridine solution. This reaction is followed by visual titration of the reaction product. The method is as follows :

Accurately weight 0.4 meq. of terpin hydrate directly into a 125 ml Erlenmeyer flask, add 4.0 ml of the dinitrobenzoyl chloride solution, stopper the flask, swirl gently to dissolve, and allow to stand 5 to 15 minutes at room temperature. Then add 7 to 10 drops of water.

Prepare a blank solution by pipetting 4.0 ml of the dinitrobenzoyl chloride solution into a flask and immediately adding 7 to 10 drops of water. Add 40 ml. of pyridine to the reaction mixture, heat nearly to boiling, cool, then titrate with 0.2N tetrabutylammonium hydroxide to the first definite and permanent red color. The titration is best performed with the titrant and solution protected from moisture and air and the tip of the buret immersed in the titrating solution. Titrate the blank in exactly the same manner.

#### 7.4 Refractometric Method

Rapaport and Solyanik (95) have developed a rapid method of refractometric determination of 23 mixtures containing anesthesin, barbamy, bromcamphor, bromisoval, camphor, antipyrine, amidopyrine, acetalsalicylic acid, barbital, codeine, salol, terpin hydrate, hexamethylenetetramine, and phenobarbital. The method described is suitable for analysis of pharmaceutical mixtures containing compounds which are insoluble in water and soluble in ethanol. A mixture of two pharmaceuticals (0.1 g) is dissolved in 1 ml ethanol and  $n_D$  of this solution is determined. One component is chemically determined in another 0.1 g of the sample. The amount of such component is calculated from formulas:

$$A = [(n - n_0) - (C \times F)] P / 100 F_1 P_1$$

$C = VT / 100$ ,  $B = VTP / P_1$  where A is weight of the first component in grams, V is the volume of 0.1N solution used for titration of the second component, T is the g. equivalent of the first compound, B is the weight of the second component (g.), n is the refractive index of the solution,  $n_0$  is that of ethanol, C is the amount of the second component in 100 ml. solution (chemically determined F and  $F_1$  are constants determined experimentally, P is total weight of the mixture and  $P_1$  is weight of sample (0.1 g.)).

#### 7.5 Determination by means of Surface tension

Izmailov and Shvartsman (96) have reported a method for determination of phenol, terpinol hydrate and menthol in some pharmaceutical preparations by means of surface tension.

The relation between surface tension and concentration was determined for the following solutions: phenol in 3N sodium chloride, thymol in a saturated solution of benzoic acid and in 1N sodium chloride; terpinol hydrate in water and in 3N sodium chloride, menthol in 2N sodium chloride, thiocol in water and 2N sodium chloride and also resorcinol in water. A method was developed for the determination by means of surface tension of small amounts of the following: phenol in mixture with glycerol (with an accuracy not less than  $\pm 3\%$ ), thymol in mixture with sodium bicarbonate and sodium benzoate (with an accuracy not less than  $\pm 5\%$ ), terpinol hydrate in mixture with thiocol,

duotal and sugar (with an accuracy not less than  $\pm 5\%$ ) and menthol in peppermint (with an accuracy not less than  $\pm 3\%$ ). The Cantor-Rebinder method for surface-tension measurements was most convenient.

## 7.6 Spectroscopic Methods

### 7.61 Colorimetric Methods

Perelmann (97) described a colorimetric method for the estimation of terpin hydrate in tablets and drug mixtures. The procedure is as follows: Dilute 1 ml of a solution of 0.25–0.3 g terpinol hydrate in 100 ml ethanol with 10 ml ethanol then treat 5 ml of such solution with 5 ml of 5% phosphomolybdate followed by 5 ml of concentrated sulphuric acid (in 1 ml portions), shaking for a while between each addition. Dilute after 30 min. with ethanol to 25 ml and compare colorimetrically with similarly prepared standards.

A spectrophotometric method of assay involving the treatment of terpin hydrate with phosphomolybdic acid under controlled conditions have been developed by Platt and James (98, 99). By using this procedure, the terpin hydrate content of pharmaceutical products may be determined by comparison with suitable reference standard.

Terpinol hydrate elixir: Dilute a 5 ml sample to 100 ml with water. To 5 ml of 5% phosphomolybdate solution, add 1.5 ml of sample and 6 ml of concentrated sulfuric acid in 2 ml portions, mixing after each addition. Cool for 15 min. and dilute to 100 ml with water. Read after 9 min. against a reagent blank at 660 nm.

Tablets: Grind twenty tablets to a fine powder. Weigh a suitable sample, add water, and let it stand for one hour with occasional agitation. Filter and dilute so that the final solution contains 0.85–2.55 mg per 100 ml as for elixir starting from "To 5 ml. of 5%..." proceed.

Another colorimetric method for the determination of terpin hydrate is presented in "Official Methods of Analysis" (100). The method is as follows:

5 ml of the sample is introduced into a distilling flask. 100 ml saturated sodium chloride solution, 35 ml alcohol, 2 ml acetic acid and 10 ml water are added. Distill, collecting 100 ml distillate. 5 ml of phosphotungstic-phosphomolybdic acid reagent is then introduced into 50 ml volumetric flask. Cool under running water while slowly adding 5 ml sulphuric acid. Let the mixture come to room temperature and then add exactly 2 ml of the sample distillate. Place flask in boiling water for 20 min. Cool under water to room temperature and dilute to mark with dilute alcohol (1+3). Shake every few min. until solution is clear (10-15 min.). Let it stand for 0.5 hr and determine the absorbance at 725 nm against a blank, prepared without sample. Also determine absorbance of standard solution prepared simultaneously with sample. Terpin hydrate (g/100 ml elixir) =  $A \times C \times 20/A'$ ; where A = sample absorbance, A' = standard absorbance, and C = g terpin hydrate/100 ml standard solution.

Mesnard and Bertucat (101) have reported a colorimetric method for the analysis of terpin hydrate in preparations containing water, ethanol or other hydroxyl compounds. The procedure involved is as follows:

Add a 0.1% aqueous solution of the sample 1 ml to a mixture of 10% ammonium molybdate 1 ml and water 2 ml. Add dioxan 1 ml and concentrated sulphuric acid 1.5 ml and compare the blue color with that of a similarly treated standard.

Vadodaria, et al., (102) have proposed a colorimetric method of assay for terpin hydrate involving the development of bluish-green colour with phosphomolybdic acid. This method is specific for terpin hydrate in the presence of other commonly occurring ingredients of cough mixtures.

Procedure: Extract the preparation containing 5 to 6 mg of terpin hydrate in water with light petroleum (25 to 30 ml). Wash the extract with water adding the washings to the aqueous solution. Extract the aqueous layer with chloroform (5 x 20 ml), combine the extracts and dilute to 100 ml with chloroform. To a 10 ml aliquot, add 10 ml of

chloroform, 5 ml of water and molybdophosphoric acid reagent. Prepare a standard containing 0.6 mg of terpin hydrate, 5 ml of water, 20 ml of chloroform and 2 ml of molybdophosphoric acid reagent. Shake each mixture for 10 min., remove the chloroform, cool in ice and add sulphuric acid 2 ml at 0°. Set aside for 30 min. at 0°, heat at 90° to 95° for 10 min., cool and dilute to 25 ml with acetone. Measure the extinction with filter 608 against a reagent blank. Results are reproducible to  $\pm 3\%$ .

#### 7.62 Infrared spectrophotometric Method

Carol (103) has reported an infrared spectrophotometric method for the detection and estimation of various drug materials in pharmaceutical preparations including terpin hydrate. Analysis of these drugs in the absence of interfering substances can be made with certainty by conventional infrared spectrophotometric procedures. The drug material in the pharmaceutical preparation is first extracted and then dissolved in a suitable solvent. The absorbance of the compound relative to the solvent is determined. Similarly the absorbance of the standard solution of the compound is also determined. Then the amount of the compound in the given sample is calculated.

#### 7.7 Chromatographic Methods

##### 7.71 Thin layer chromatography

A thin-layer chromatographic method for the analysis of monoterpenes including terpin hydrate was reported (104). A 0.25 mm. silica gel G layer was used with n-hexane, or n-hexane containing 15% ethyl acetate as solvent. The spray reagent was concentrated sulphuric acid or phosphoric acid, antimony pentachloride in carbon tetrachloride antimony trichloride in chloroform or potassium permanganate.  $R_f$  values are given.

##### 7.72 Gas Liquid Chromatography

A GLC method for the identification of terpin hydrate has been carried out in our laboratory (105), using a Varian GC-3700 gas chromatograph equipped with Varian CDS 111 integrator.

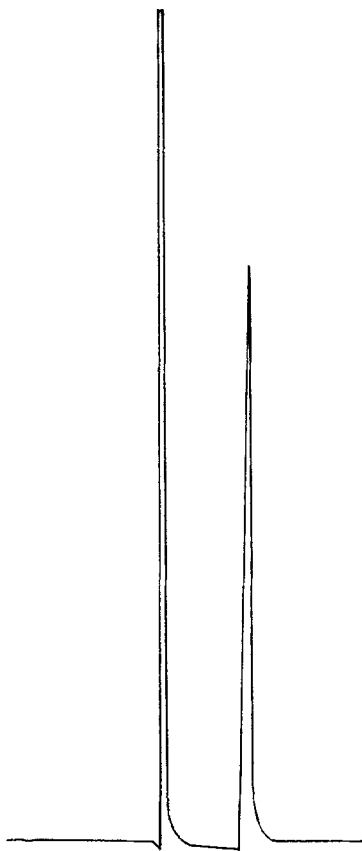


Fig. 9: GLC of Terpin hydrate.

Column condition : 3% OVI on Gas Chrom Qi glass column (2 m x 2 mm). The column run isothermally at 110°C; carrier gas : Nitrogen, flow rate was adjusted to 40 ml/min. Detector : FID at 250°C, hydrogen and air flow rate were adjusted to 30 ml/min. and 300 ml/min. respectively. Methanol was used as solvent. Sample size was 1  $\mu$ l. The injection temperature was 150° and the chart speed was adjusted to give 1 cm/min. The retention time = 2.5 min. The GLC of terpin hydrate is presented in Fig. 9.

The National Formulary XIV (18) describes a GLC method for the determination of terpin hydrate in commercial terpin hydrate as well as terpin hydrate elixirs.

Assay: a) For terpin hydrate

Internal standard solution: Dissolve about 1g of dodecyl alcohol, accurately weighed, in sufficient dehydrated alcohol to make 100 ml.

Standard preparation:-. Transfer about 40 mg of NF terpin hydrate reference standard, accurately weighed, to a 10 ml volumetric flask, and dissolve in about 6 ml of dehydrated alcohol. Add 2.0 ml of internal standard solution, dilute to volume with dehydrated alcohol, and mix.

Assay preparation:-. Transfer about 200 mg of terpin hydrate, accurately weighed, to a 50-ml volumetric flask, and dissolve in about 30 ml of dehydrated alcohol. Add 10.0 ml of internal standard solution, dilute to volume with dehydrated alcohol, and mix.

Calibration:- Inject an appropriate volume of the standard preparation into a suitable gas chromatograph equipped with a flame-ionization detector. Under typical conditions, the instrument contains a 1.8 m x 3-mm stainless steel column packed with 10% polyethylene glycol 20,000 on 60 to 80-mesh, silanized chromatographic siliceous earth. The column is maintained at 170°, the injection port and the detector are maintained at 170°, and dry helium is used as the carrier gas at a flow rate of 40 ml per minute. In the detector, hydrogen is introduced at a rate of 44 ml per minute, and air at a rate of 360 ml per minute.

Measure the heights of the first (dodecyl alcohol) and second (terpin hydrate) peaks, and record the values as  $P_1$  and  $P_2$ , respectively. Calculate the relative response factor,  $F$ , of equal weights of dodecyl alcohol and NF Terpin Hydrate Reference Standard by the formula:

$$(P_1/P_2)(W_2/W_1)$$

in which  $W_2$  represents the weight percentage of NF terpin hydrate reference standard in the standard preparation, calculated on the anhydrous basis, and  $W_1$  represents the weight percentage of dodecyl alcohol in the standard preparation.

Procedure:- Inject an appropriate volume of the assay preparation into the chromatograph, and obtain a chromatogram under the conditions described for Calibration. Measure the heights of the first (dodecyl alcohol) and second (terpin hydrate) peaks, and record the values as  $p_1$  and  $p_2$  respectively. Calculate the weight, in mg, of  $C_{10}H_{20}O_2$  in the terpin hydrate taken by the formula:

$$LFC (p_2/p_1)$$

in which F is as defined under Calibration, and C is the exact concentration, in mg per ml, of the internal standard solution.

#### b) For terpin hydrate elixirs

Procedure:- Transfer an accurately measured volume of terpin hydrate elixir, equivalent to about 170 mg of terpin hydrate to a separator, add 25 ml of a standard solution of sodium acetate and shake for 1 minute and allow to stand for 30 minutes. Then extract with 25 ml. portions of chloroform. Evaporate the chloroform extract to dryness without heating. Dissolve the residue 15 ml of dehydrated alcohol and transfer to a volumetric flask containing 10 ml of internal standard solution. Then proceed as directed in the assay above under terpin hydrate.

Many GLC methods for determination of terpin hydrate in pure form as well as in pharmaceutical preparations have been reported (106-112). The methods involve the either direct chromatographic comparison of terpin hydrate and the internal standard or the formation and chromatographic measurement of the corresponding trimethylsilyl (TMS) ether derivatives. Table 5 shows the various systems that have been used for the analysis of terpin hydrate. The chromatograms of some of the above methods are also presented (Figs: 10,11).



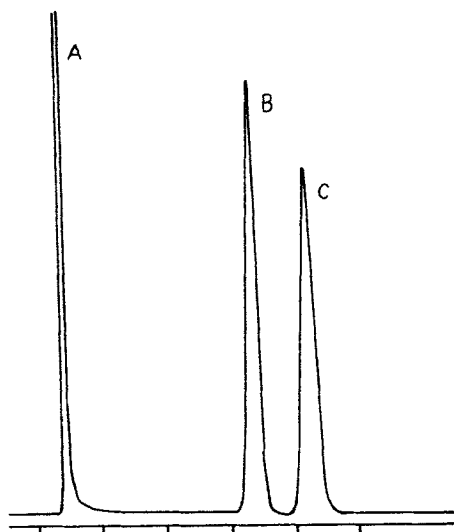


Fig. 10: Chromatogram of Terpin hydrate and 3-tert-Butylphenol.

(A : Solvent; B : Terpin hydrate; C : 3-tert-Butylphenol).

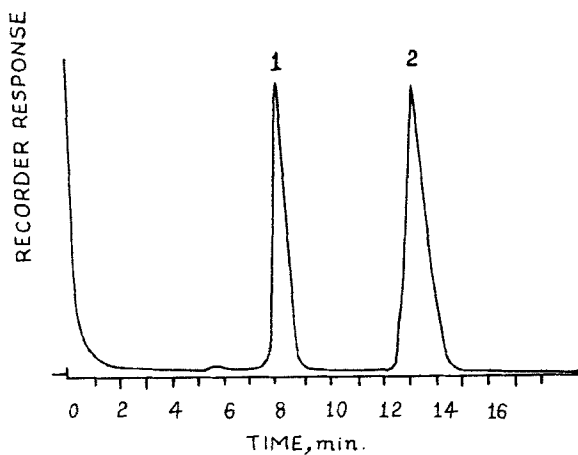


Fig. 11: Chromatogram of Terpin hydrate and dodecyl alcohol.

(1. Dodecyl alcohol; 2. Terpin hydrate.)

Table 5 : GLC conditions used for Terpin Hydrate.

S. No.	Column	Support	Liquid Phase	Carrier gas	Flow rate	Internal standard	Temperature.	Detector	Ref.
1.	Stainless-steel 6 ft x 1/8 in.	Diatoport-S 60/80 mesh.	10% carbowax 20 M.	Helium	40 ml/min	Dodecyl alcohol.	170°	FID	107
2.	Stainless-steel 6 ft x 1/8 in.	Diatoport-S 60/80 mesh.	10% SE-30	Helium	65 ml/min	Dodecyl alcohol.	140°	FID	107
3.	Stainless-steel 6 ft x 1/8 in.	Diatoport-S 60/80 mesh	10% SE-30	Helium	40 ml/min or 65 ml/min	Dodecyl alcohol	170° or 140°	FID	108
4.	Borosilicate glass 5 ft x 3 mm	Chromosorb-W 60/80 mesh.	Carbowax 20 M terephthalic acid	Nitrogen	40 ml/min.	Biphenyl	160°	FID	109
5.	Glass 1.2 x 3 mm	Diatoport-S 80/100 mesh	5% hydrogenated Castor oil (Castrowax).	Helium	60 ml/min	3-tert- butylphenol	140°	FID	110
6.		Chromosorb W	SE-30, with neo- pentyl glycol succinat polyester	-	-	-	-	-	111
7.	Glass 1.5 m x 4 mm	Chromosorb G 80/100 mesh.	5% Apiezon L on A.W.-DMES	Nitrogen	50 ml/min	Menthol	170°	FID	112

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# ATROPINE

Abdullah A. Al-Badr and Farid J. Muhtadi

*King Saud University*

*Riyadh, Saudi Arabia*

1. Description	326
1.1 Nomenclature	326
1.2 Formulae	326
1.3 Molecular Weight	328
1.4 Elemental Composition	328
1.5 Appearance, Color, Odor, and Taste	328
1.6 Dissociation Constant	328
1.7 pH range	328
2. Physical Properties	329
2.1 Melting Point	329
2.2 Sublimation Range	329
2.3 Solubility	329
2.4 X-Ray Crystallography	329
2.5 Spectral Properties	330
3. Isolation	340
4. Synthesis	340
4.1 Partial Synthesis	340
4.2 Total Synthesis	340
5. Biosynthesis	352
5.1 Biosynthesis of Tropine	352
5.2 Biosynthesis of Tropic Acid	354
6. Metabolism	355
7. Pharmacokinetics	357
8. Therapeutic Uses of Atropine	358
9. Methods of Analysis	359
9.1 Identification Tests	359
9.2 Microcrystal Tests	360
9.3 Titrimetric Methods	360
9.4 Polarographic Methods	365
9.5 Spectrophotometric Methods	366
9.6 Chromatographic Methods	373
9.7 Radio-immunoassay	378
References	380

# 1. Description

## 1.1 Nomenclature

### 1.1.1 Chemical Names

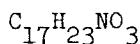
- a) endo ( $\pm$ )- $\alpha$  -(Hydroxymethyl) benzene-acetic acid 8-methyl-8-azabicyclo [3.2.1] oct-3-yl ester.
- b) Benzene-acetic acid  $\alpha$ -(hydroxymethyl)-, 8-methyl-8-azabicyclo [3.2.1] oct-3-yl ester, endo-( $\pm$ )-
- c) 1 $\alpha$  H,-5 $\alpha$  H-tropan-3 $\alpha$  -ol( $\pm$ )-tropate.

### 1.1.2 Generic Names

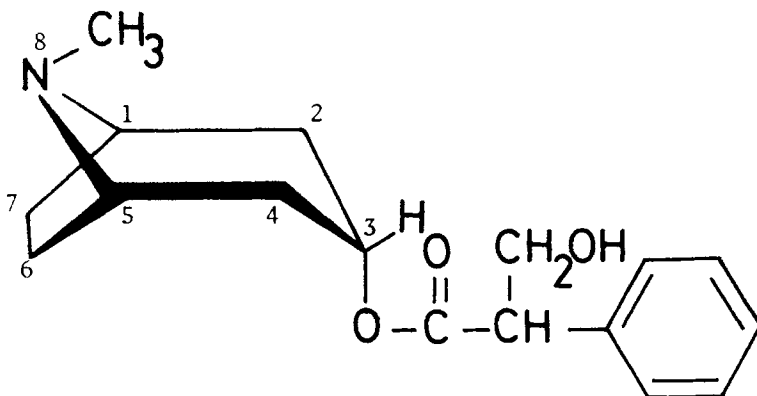
Atropine, dl-hyoscyamine, ( $\pm$ )-hyoscyamine, tropic acid ester with tropine, tropine ( $\pm$ ) tropate, dl-tropanyl tropate, ( $\pm$ ) tropanyl tropate.

## 1.2 Formulae

### 1.2.1 Empirical



### 1.2.2 Structural



The structure was confirmed by the total synthesis of atropine which was achieved by several authors (1-4).

1.2.3 CAS Registry No.

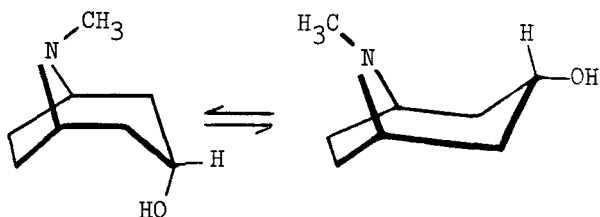
[51-55-8]

1.2.4 Wiswesser Line Notation

T56 A ANTJ A  
-GOVYR & 1Q \_ DL (5)

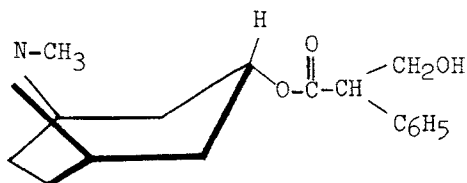
1.2.5 Stereochemistry

Examination of the NMR spectra of some tropane deuteriohalides has shown that the N-substituent in tropanes is predominantly equatorial (6). X-ray analysis of tropine hydrobromide has shown the presence of chair conformation (7). Study of the dipole-moment and Kerr-constant measurements of a number of tropane derivatives has shown that the piperidine ring is in the chair form with the N-methyl equatorial (8). Another study of the dipole-moments and NMR spectra of some tropane derivatives have confirmed that the piperidine ring is in the chair conformation with the N-methyl group predominantly equatorial (9). In tropine, however, the predominant conformation is the piperidine ring in a deformed chair form together with a minor amount in the boat form (10).



Tropine

In atropine, the  $\alpha$ -3-substituent is of greater bulk than the hydroxyl, and the boat form may well be favored because of the increased interactions involving the dimethylene bridge in the chair confirmation (11).



A detailed review is available for the boat or chair conformation in tropines (12).

Other PMR study suggested a preference for the boat conformation in several tropane derivatives. This study showed strong cross-ring intramolecular interactions of the type  $N \cdots C=O$  and  $N \cdots H-O$  were indicated by the broadening of the proton signal due to the coupling between 1(5)-H and 2(4)-H protons in the boat conformer compared with the chair. This broadening arises as a consequence of eclipsing of these protons in the boat conformer (13). Carbon-13 magnetic resonance study has also suggested a non-chair conformations in tropane derivatives (14).

### 1.3 Molecular Weight

289.38

### 1.4 Elemental Composition

C, 70.56%; H, 8.01%; N, 4.84%; O, 16.59%

### 1.5 Appearance, Color, Odor and Taste

Colorless needle-like crystals or white crystalline powder, odorless and has a sharp bitter taste.

### 1.6 Dissociation Constant

pKa 5.93

### 1.7 pH range

pH of 0.0015 molar solution is 10.0 (15), approximate pH of saturated aqueous solution is 9.5 (16).

## 2. Physical Properties

### 2.1 Melting Point

114 - 116° (15)

114 - 118° (16)

### 2.2 Sublimation range

Atropine sublimes in high vacuum at 93-110°.

### 2.3 Solubility

One gram dissolves in 460 ml water, in 90 ml water at 80°, in 2 ml alcohol, 1.2 ml alcohol at 60°, in 27 ml glycerol, 25 ml ether. Soluble in benzene and dilute acids.

### 2.4 X-ray crystallography

The X-ray crystallography of tropine hydrobromide (7), tropine ethobromide (17) pseudotropine (18), hyoscine hydrobromide (19) and tropic acid in hyoscine N-oxide (20) have been reported.



## 2.5 Spectral Properties

### 2.5.1 Ultraviolet Spectrum

The UV spectrum of atropine in ethanol (Fig.1) was scanned from 200 to 400 nm using DMS 90 Varian Spectrophotometer. It exhibited the following UV data (Table 1).

Table 1. UV characteristics of atropine

<u><math>\lambda</math> max. at nm</u>	<u><math>\epsilon</math></u>	<u>A(1%, 1 cm)</u>
205	-	-
246	147.6	5.1
251.5	175.1	6.05
257	209.8	7.25
263.5	143.3	4.95
271	24.6	0.85

Other reported UV spectral data for atropine in 0.1 N sulfuric acid (21):

$\lambda$  max at. 252 m $\mu$  (E 1%, 1 cm 5), 258 m $\mu$  (E 1%, 1 cm 6) and 264 m $\mu$  (E 1%, 1 cm 5).

### 2.5.2 Infrared Spectrum

The IR spectrum of atropine as KBr-disc was recorded on a Perkin Elmer 580 B Infrared Spectrophotometer to which Infrared Data station is attached (Fig. 2).

The structural assignments have been correlated with the following frequencies (Table 2).

Table 2. IR Characteristics of Atropine

<u>Frequency cm<sup>-1</sup></u>	<u>Assignment</u>
3070	OH (hydrogen bonded)
2930	CH (stretch)
2810	N-CH <sub>3</sub>
1725	$\begin{array}{c} \text{O} \\ \parallel \\ \text{O}-\text{C} \end{array}$ - (ester)
1595, 1580	C=C aromatic

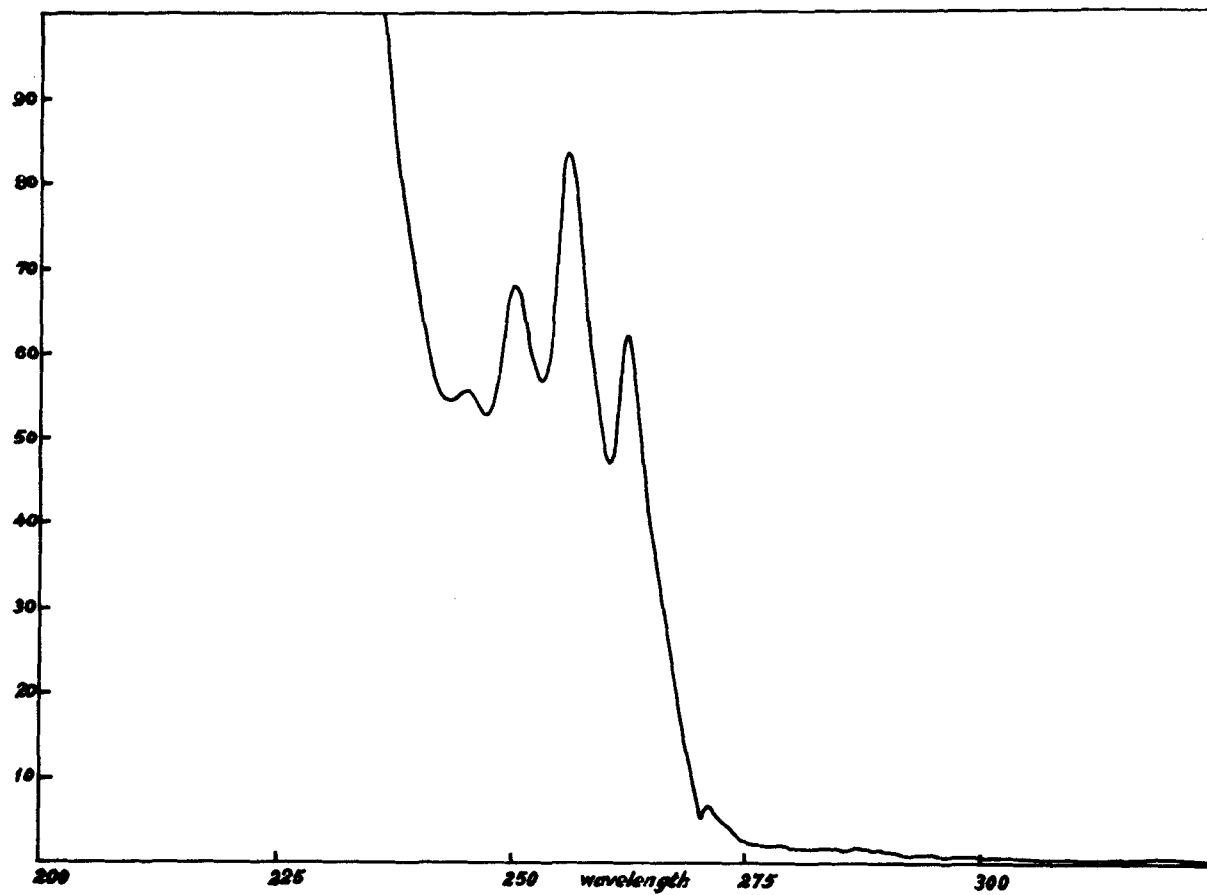


FIG. 1. THE UV SPECTRUM OF ATROPINE IN ETHANOL

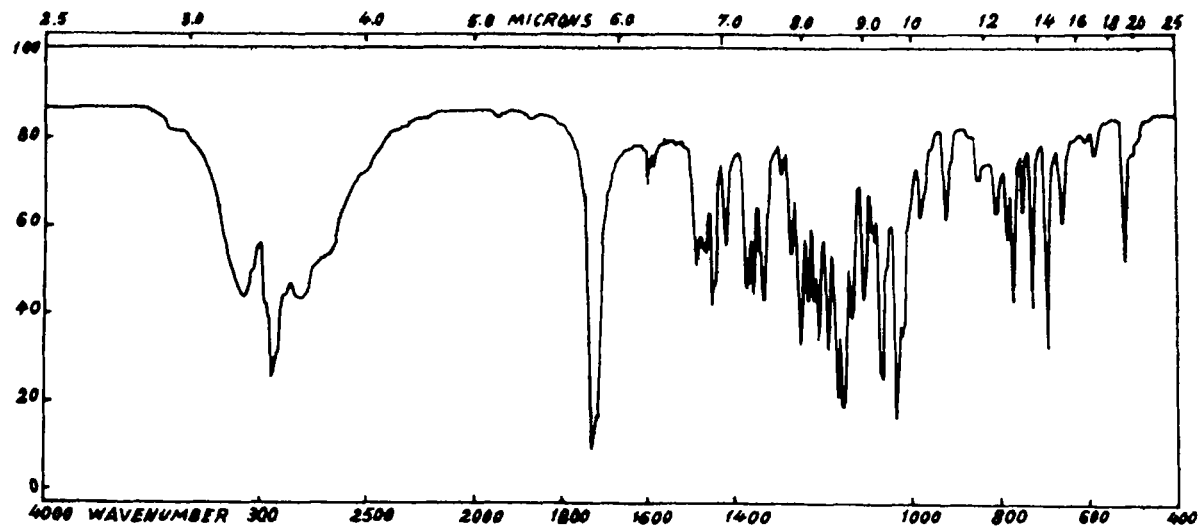


FIG. 2. THE IR SPECTRUM OF ATROPINE AS KBr-DISC

<u>Frequency cm<sup>-1</sup></u>	<u>Assignment</u>
1155, 1030	C-O-C (ether)
770, 725, 690	5H (mono substituted aromatics)

The IR exhibited the following other characteristic bands:-

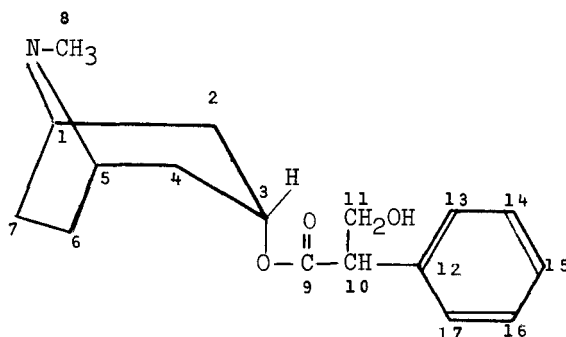
1450, 1420, 1370, 1355, 1335, 1270, 1245, 1230, 1220, 1205, 1190, 1165, 1132, 1108, 1065, 975, 920, 845, 805, 515 cm<sup>-1</sup>.

Other IR data for atropine (5,21) have been also reported.

### 2.5.3 Nuclear Magnetic Resonance Spectra

#### 2.5.3.1 Proton Spectra

The PMR spectra of both atropine in CDCl<sub>3</sub> and in TFA (Trifluoroacetic acid) were recorded on a Varian T-60A, 60 MHz NMR Spectrometer using TMS (Tetramethylsilane) as an internal reference. These are shown in Fig. 3(a) and 3(b) respectively. The following structural assignments have been made (Table 3).



Other PMR data for atropine are also reported (5,9, 13,22).

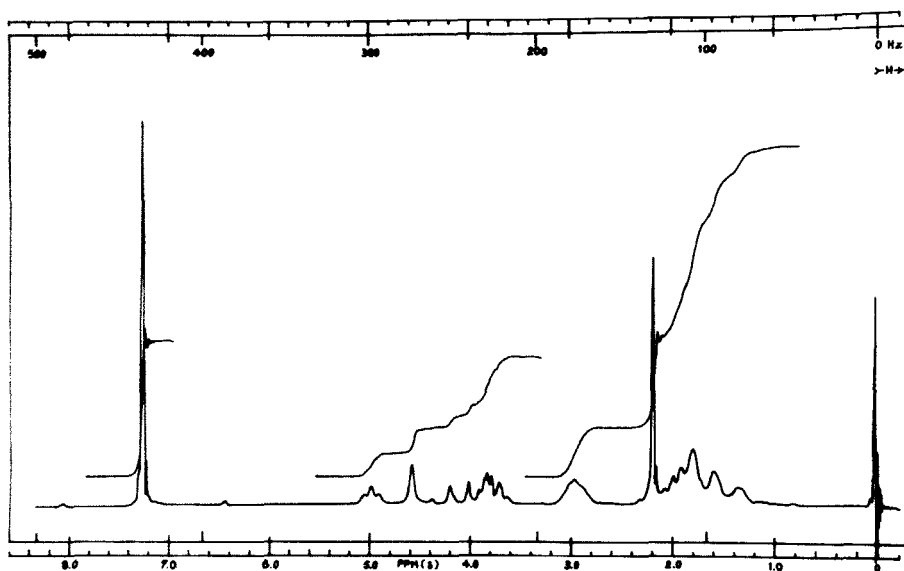
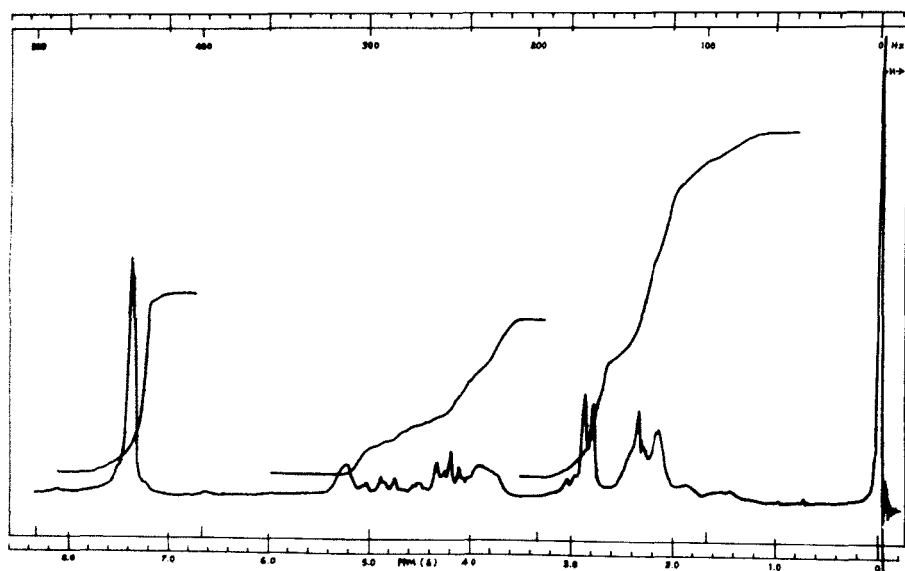
FIG. 3(A). THE PMR SPECTRUM OF ATROPINE IN  $\text{CDCl}_3$ FIG. 3(B). THE PMR SPECTRUM OF ATROPINE IN  $\text{TFA}$ .

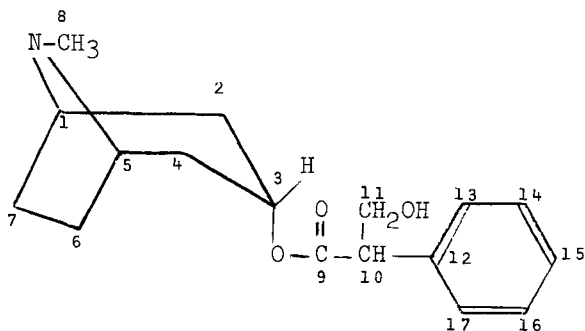
Table 3. PMR characteristics of atropine

Group	Chemical Shift (ppm)	
	$\text{CDCl}_3$	TFA
5 aromatic protons 13,14,15,16,17	7.23(s)	7.36(s)
3 H	4.96(t)	5.26(bs)
$\text{CH}_2 - \text{OH}$	4.56(s)	4.86(m)
$\text{CH}_2 - \text{OH}$ , 10 $\text{CH}$	3.9(m)	4.23(m)
1,5 H	2.93(bs)	3.86(bs)
8-N-Me	2.16(s)	2.85(d)
2,4,6,7 H	1.66(m)	2.26(m)

s=singlet, d=doublet, t=triplet, bs=broad singlet, m=multiplet

### 2.5.3.2 $^{13}\text{C}$ -NMR

The  $^{13}\text{C}$ -NMR noise decoupled and off resonance spectra are presented in Fig. 4 and Fig. 5 respectively. Both were recorded over 4000 Hz range in deuterated chloroform on a Varian FT 80 A-80 MHz spectrometer, using 10 mm sample tube and tetramethyl silane as a reference standard at 21°. The carbon chemical shifts are assigned on the bases of the additivity principals and off resonance splitting pattern (Table 4).



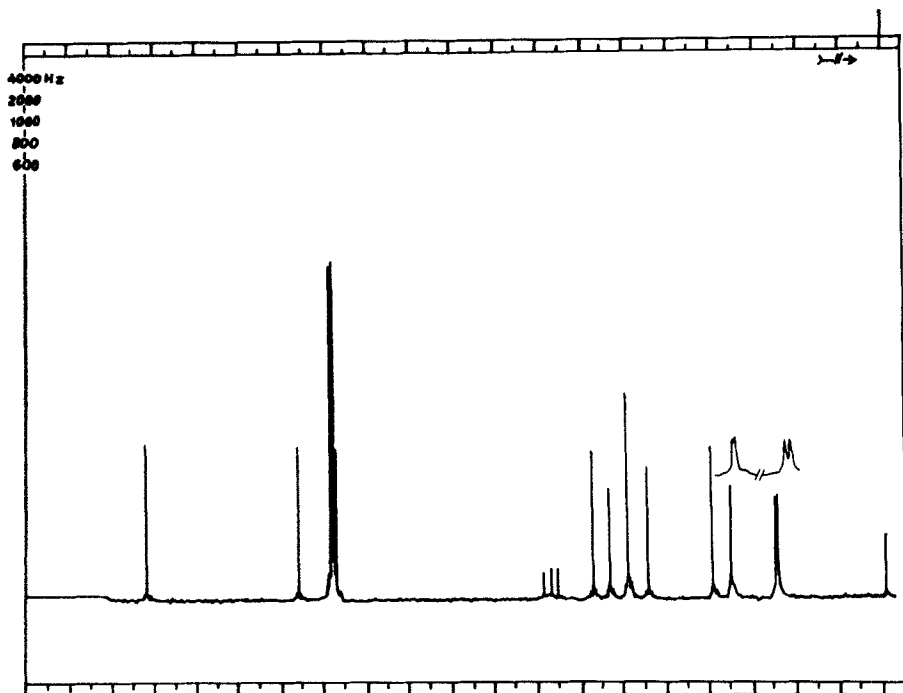
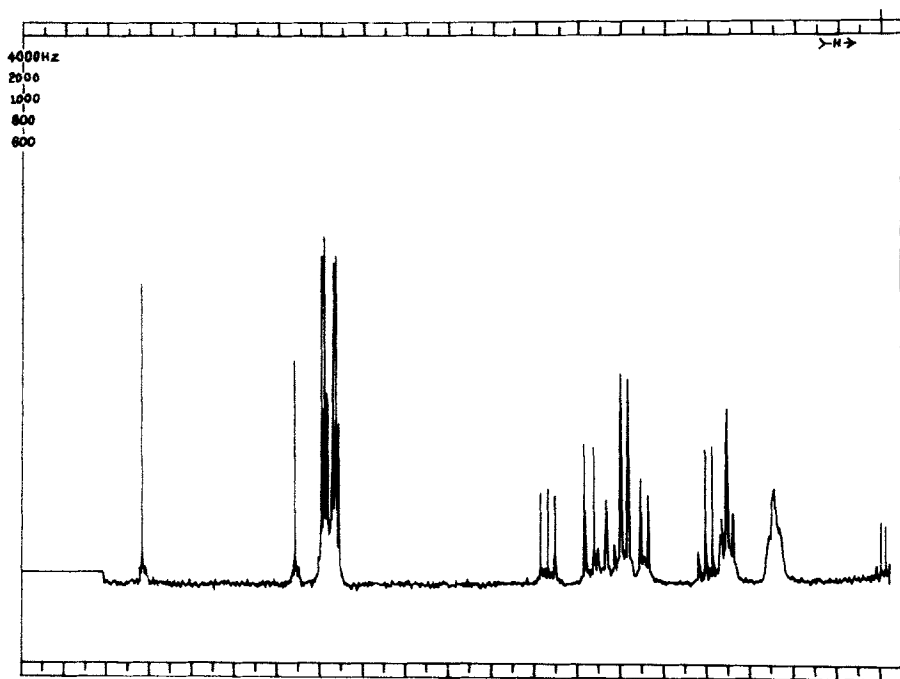
FIG. 4. THE  $^{13}\text{C}$ -NMR NOISE DECOUPLED SPECTRUM OF ATROPINEFIG. 5.  $^{13}\text{C}$ -NMR OFF RESONANCE SPECTRUM OF ATROPINE

Table 4. Carbon Chemical Shifts of Atropine

Carbon no.	Chemical Shift [ppm]	Carbon no.	Chemical Shift [ppm]
C <sub>9</sub>	171.92(s)	C <sub>1</sub> , C <sub>5</sub>	59.54(d)
C <sub>12</sub>	136.17(s)	C <sub>10</sub>	54.94(d)
C <sub>13</sub> , C <sub>17</sub>	128.66(d)	C <sub>8</sub>	40.08(q)
C <sub>14</sub> , C <sub>16</sub>	128.11(d)	C <sub>2</sub> , C <sub>4</sub>	36.04(t)
C <sub>15</sub>	127.48(d)	C <sub>7</sub>	25.31(t)
C <sub>3</sub>	67.63(d)	C <sub>6</sub>	24.93(t)
C <sub>11</sub>	63.54(t)		

s=singlet, d=doublet, t=triplet, q=quartet.

Other <sup>13</sup>C-NMR data for atropine (14,23), atropine hydrochloride (14) and atropine methiodide (14) have also been reported.

#### 2.5.4 Mass Spectrum

The mass spectrum of atropine is presented in Fig. 6. This was obtained by electron impact ionization on a Varian MAT 1020 by direct inlet probe at 270°C. The electron energy was 70 eV. The spectrum scanned to mass 300 amu. The spectrum (Fig. 6) shows a molecular ion peak M<sup>+</sup> at m/e 289 with relative intensity 9.50%. The base peak is 124 with relative intensity 100%. The most prominent fragments, their relative intensities and some proposed ion fragments are given in table 5.



MASS SPECTRUM  
12/23/84 12:01:00 + 35:53  
SAMPLE: KSV SAMPLE ATROPINE + INT STD 90-2700  
ENHANCED (S 15B 2N)

DATA: KSV #1389

BASE M/E: 124  
RIC: 267264.

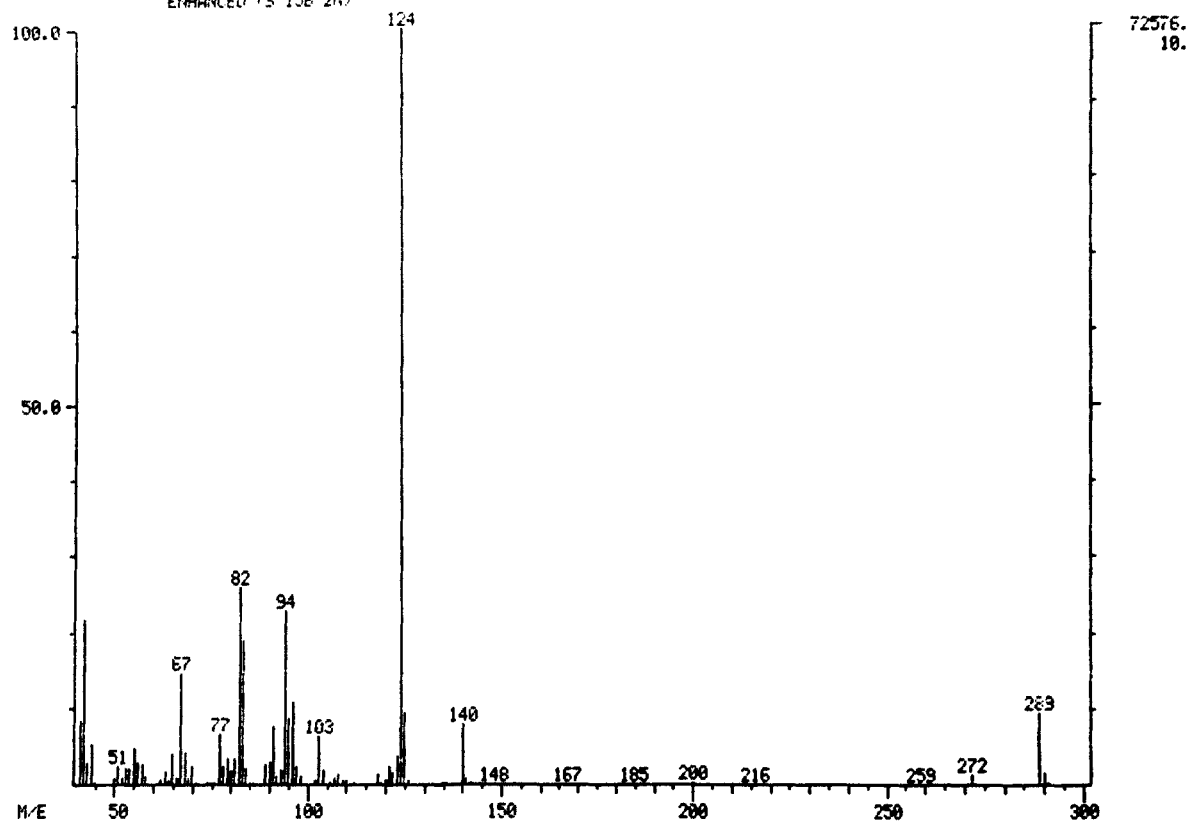
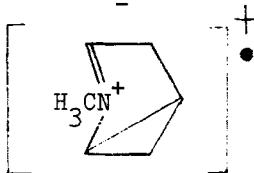
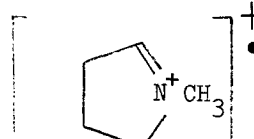
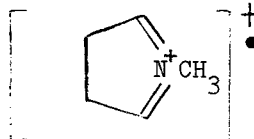
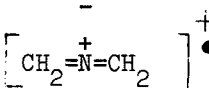


FIG. 6. THE MASS SPECTRUM OF ATROPINE.

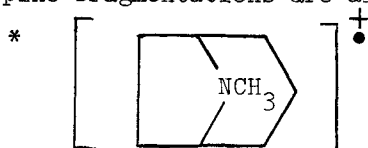
Table 5. Mass Fragments of Atropine

<u>m/e</u>	<u>Relative intensity %</u>	<u>Ions</u>
289	9.50	M <sup>+</sup>
140	7.79	-
125	9.34	See below*
124	100.00	125-H
103	6.35	-
96	10.67	
95	8.60	96-H
94	22.66	95-H
83	18.87	
82	25.97	
67	14.78	-
44	5.15	-
42	21.83	
41	8.36	42-H

Other reported mass spectra of atropine (24):

Base peak 124, m/e: 42, 55, 67, 82, 94, 104, 124, 140, 272, 289.

Tropine fragmentations are also reported ( 25 ).



### 3. Isolation of Atropine

Atropine occurs in several solanaceous plants these include species of *Atropa*, *Datura*, *Hyoscyamus*, *Duboisia*, *Mandragora* and *Scopolia* (26). It is claimed that atropine does not occur as such in the plants, but *l*-hyoscyamine present in plants, (27) and during extraction process, *l*-hyoscyamine undergoes racemization to give atropine. *Hyoscyamus muticus* from Egypt is the preferred source for the manufacture of atropine because of its high alkaloid content, with stramonium next in order (28).

One of the best methods for the isolation of atropine is as follows (28).

The powdered drug is thoroughly moistened with an aqueous solution of sodium carbonate and extracted with ether or benzene. The alkaloidal bases are extracted from the solvent with water acidified with acetic acid. The acid solution is then shaken with ether as long as the latter takes up coloring matters. The alkaloids are precepitated with sodium carbonate, filtered off, washed and dried. The dried precipitate is dissolved in ether or acetone, dehydrated with anhydrous sodium sulfate and filtered. The filterate is concentrated, cooled, when crude hyoscyamine and atropine crystallize from the solution. The crude crystalline mass resulted is filtered off and dissolved in alcohol, sodium hydroxide solution is added and the mixture is allowed to stand until racemization of hyoscyamine to atropine is completed (as indicated by the absence of optical activity).

The crude atropine is purified by crystallisation from acetone.

### 4. Synthesis of Atropine

#### 4.1 Partial Synthesis

Landenburg in 1879 (1) accomplished the first synthesis of atropine from tropine and tropic acid, thus proving atropine to be the tropine ester of tropic acid. Tropine and tropic acid are heated in the presence of hydrogen chloride to give atropine.

#### 4.2 Total Synthesis

Since atropine is the tropine ester of tropic

acid, schemes for the total synthesis of tropine and the total synthesis of tropic acid were reported.

#### 4.2.1 Total Synthesis of Tropine

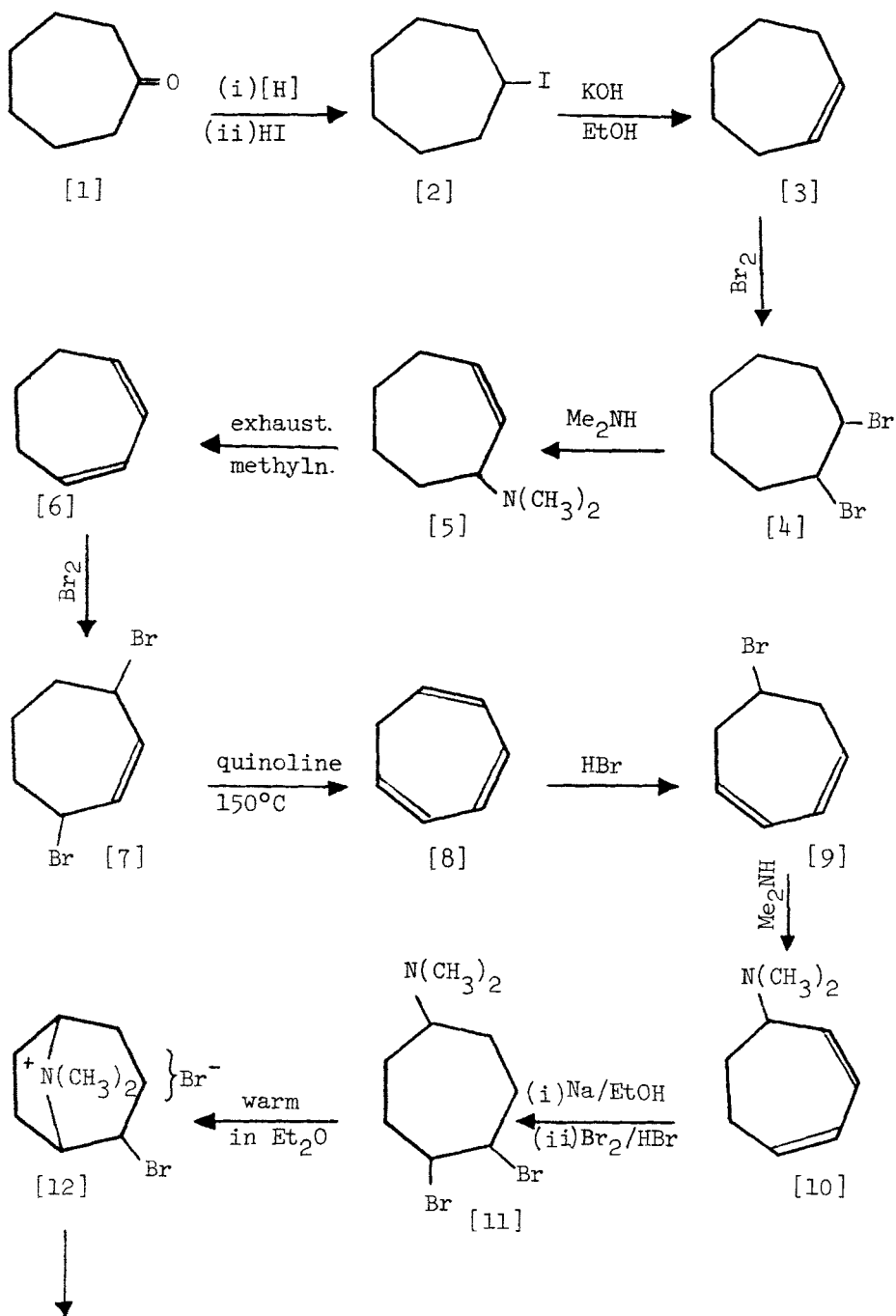
Four schemes for the total synthesis of tropine are known. Scheme II was also modified to give a much better yield.

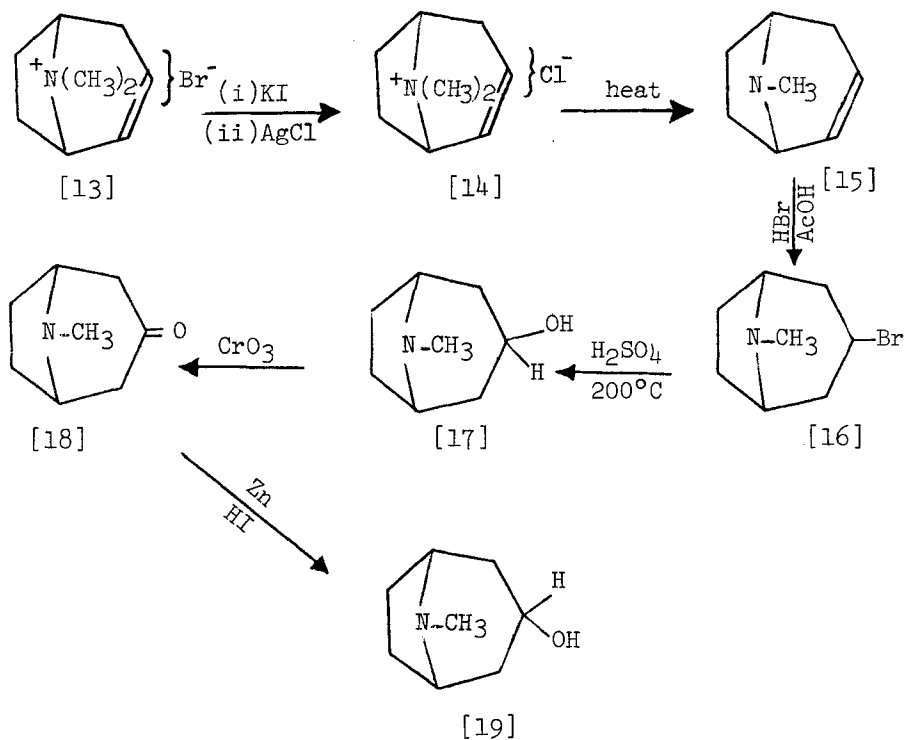
Scheme I: Willstätter's total synthesis of tropine (2).

Suberone (cycloheptanone) [1] is reduced to suberol which is treated with hydrogen iodide to give suberyl iodide [2]. This is treated with potassium hydroxide in ethanol to give cycloheptene [3]. Cycloheptene is brominated to give 1,2-dibromocycloheptane [4] which is treated with dimethylamine to yield dimethylaminocyclohept-2-ene [5]. The latter is converted to cyclohepta-1,3-diene [6] by exhaustive methylation. [6] is brominated at 1,4-positions to give 1,4-dibromocyclohept-2-ene [7]. Elimination of two moles of the hydrogen bromide of [7] is effected by quinoline to give cycloheptatriene [8].

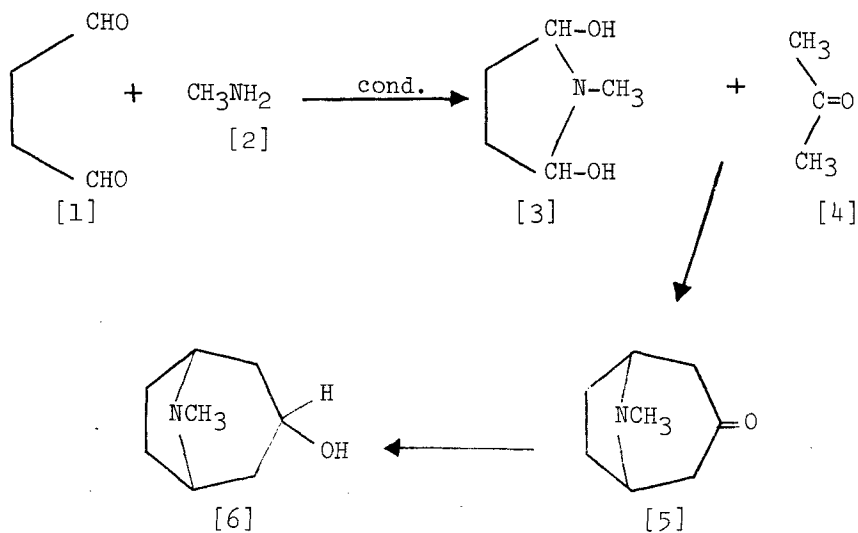
Substance [8] is treated with hydrogen bromide to give bromocyclohepta-3,5-diene [9] which is reacted with dimethylamine to give dimethyl aminocyclohepta-2,4-diene [10]. The latter is treated with sodium in ethanol followed by bromination to give 1,2-dibromo-5-dimethylamino-cycloheptane [11]. This is warmed in ether when intramolecular alkylation occurs to give 2-bromotropane methobromide [12]. Hydrogen bromide is eliminated from [12] by the action of alkali to yield tropidine methobromide [13]. This is transformed to tropidine methochloride [14] by the action of potassium iodide followed by the action of silver chloride. Substance [14] is pyrolyzed to give tropidine [15].

Hydrogen bromide is added to an acetic acid solution of tropidine [15] to yield 3-bromotropane [16] which is hydrolysed with 10% sulfuric acid at 200-210° to give pseudotropine [17].  $\psi$ -tropine [17] is oxidized with chromium trioxide to give tropinone [18].

Scheme I: Willstätter's total synthesis of tropine



Scheme II: Robinson's total synthesis of tropine



This ketone is reduced with zinc and hydriodic acid to tropine [19].

Scheme II: Robinson's synthesis (3)

Succindialdehyde [1] is condensed with methylamine [2] to give the condensate biscarbinolamine [3]. This in turn condensed with acetone [4] to give tropinone [5] (This mixture is allowed to stand in water at ordinary temperature for half an hour).

Tropinone [5] is reduced with zinc and hydriodic acid to tropine [6].

The yield can be improved by substitution of the more reactive acetone dicarboxylate or its ester for acetone.

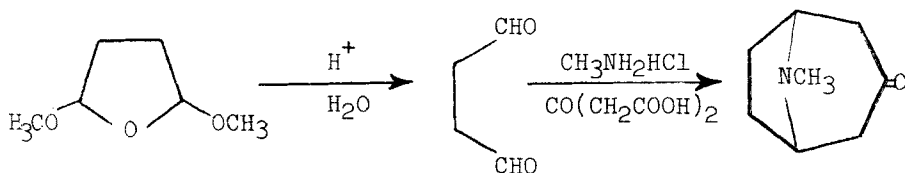
Succindialdehyde [1] is condensed with methylamine [2] to give biscarbinolamine [3]. [3] is condensed with calcium acetonedicarboxylate [4] to afford the condensate [5]. This is warmed with hydrochloric acid to give tropinone [6]. Tropinone [6] is reduced with zinc and hydriodic acid to tropine [7].

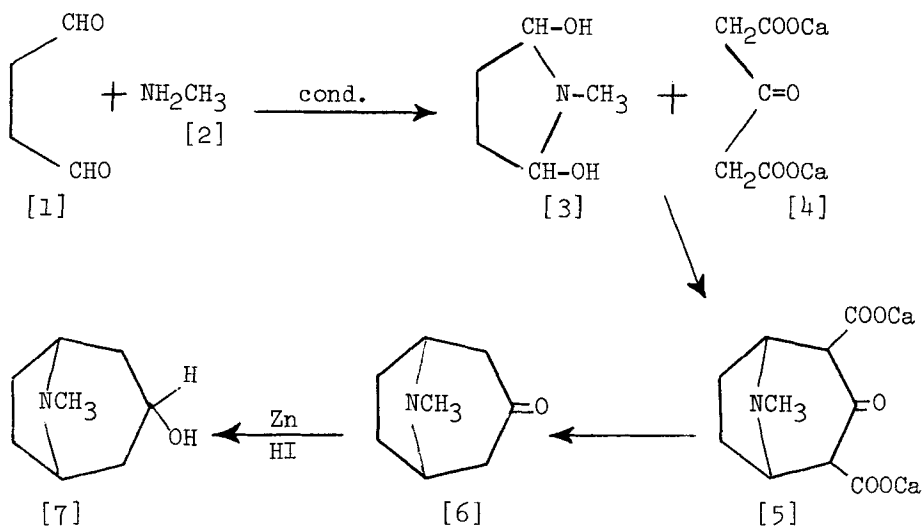
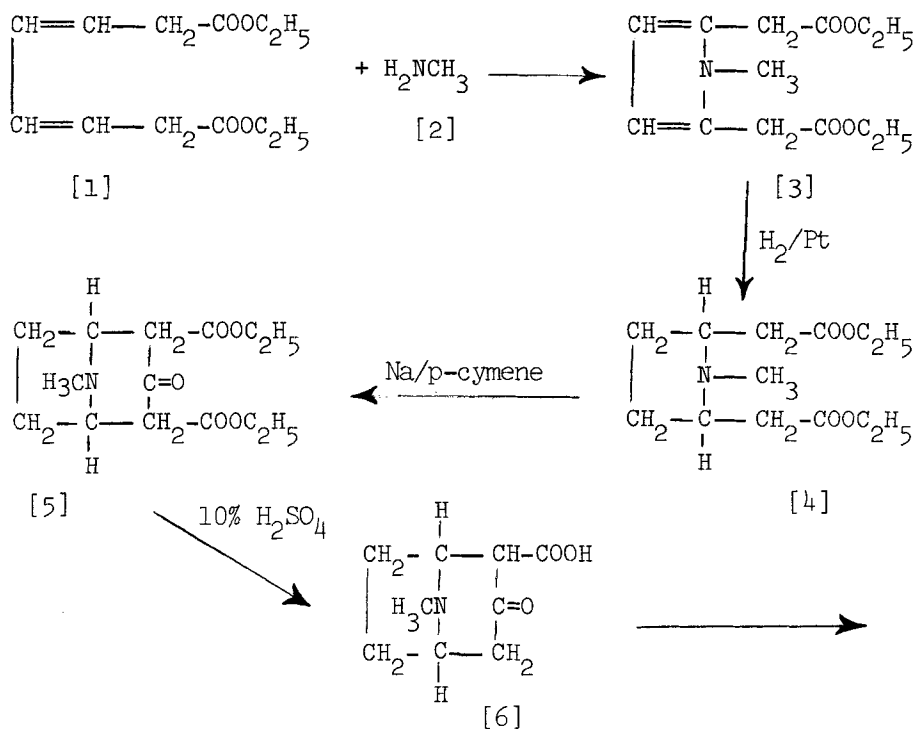
Scheme III: Willstatter's second synthesis(4)

Succinylodiacetic ester [1] is condensed with methylamine [2] to give diethyl-N-methylpyrrolediacetate [3]. This is reduced ( $H_2+Pt$ ) to afford diethyl-N-methylpyrrolidinediacetate [4]. The cis form of [4] is cyclized in the presence of Na and p-cymene to give ethyltropinone-2-carboxylate [5]. Hydrolysis of [5] with 10% sulfuric acid gives ethyltropinone-2-carboxylic acid [6]. The latter is heated to yield tropinone [7] which is reduced with zinc and hydriodic acid to tropine [8].

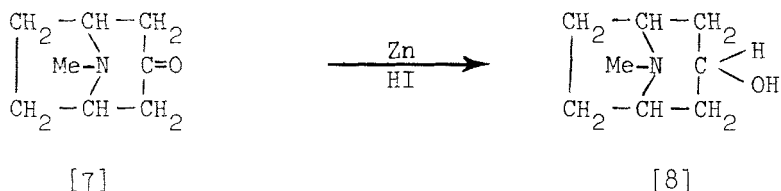
Scheme IV:

Tropinone can also be synthesized (29) using methylamine hydrochloride, acetondicarboxylic acid and generating succindialdehyde in situ by the action of acid on 2,5-dimethoxy tetrahydrofuran as follows:



Scheme II: Robinson's synthesis (yield improvement)Scheme III: Willstätter's second synthesis





#### 4.2.2 Total Synthesis of Tropic acid

Several schemes for the total synthesis of tropic acid are known (Scheme I to V).

Scheme I: Landenburg's synthesis (30).

Acetophenone [1] is converted into  $\alpha,\alpha$ -dichloroethylbenzene [2] by the action of phosphorous pentachloride. [2] is reacted with potassium cyanide and ethanol to furnish  $\alpha$ -ethoxy- $\alpha$ -cyanoethylbenzene [3]. This is hydrolysed with barium hydroxide solution to give atrolactic ethylether [4]. The latter is heated with hydrogen chloride to yield atropic acid [5] which is converted to tropic acid [6].

Scheme II: McKenzie and Wood's synthesis (31).

Acetophenone [1] is converted by the action of potassium cyanide to acetophenone cyanohydrine [2]. This upon hydrolysis is converted into atrolactic acid [3]. The latter is heated under pressure to yield atropic acid [4]. Atropic acid [4] is treated with hydrogen chloride in ethereal solution to form  $\beta$ -chlorohydratropic acid [5]. This upon boiling with aqueous sodium carbonate is changed to tropic acid [6].

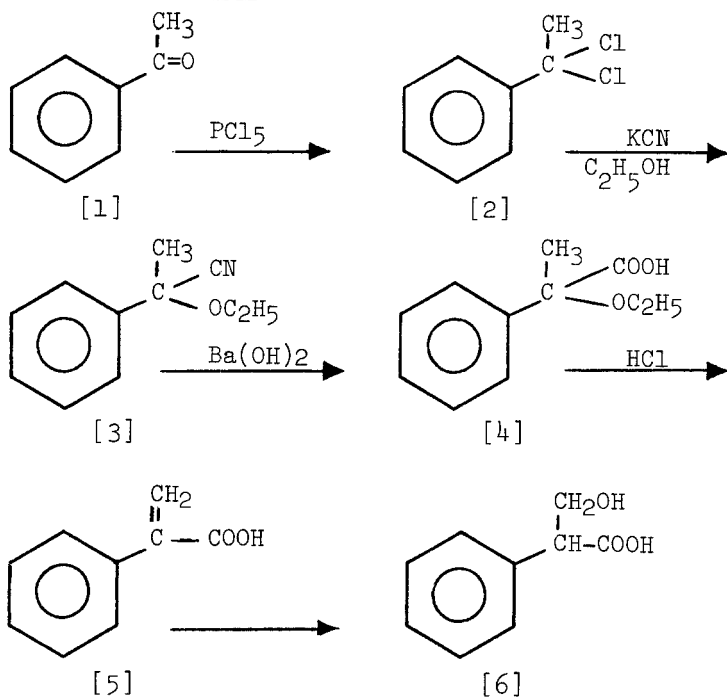
Scheme III: Muller's synthesis (32).

Ethylphenyl acetate [1] is condensed with ethylformate to give ethyl  $\alpha$ -formyl acetate [2]. This on reduction with aluminium amalgam yields dl-tropic ester [3] which upon hydrolysis gives tropic acid [4].

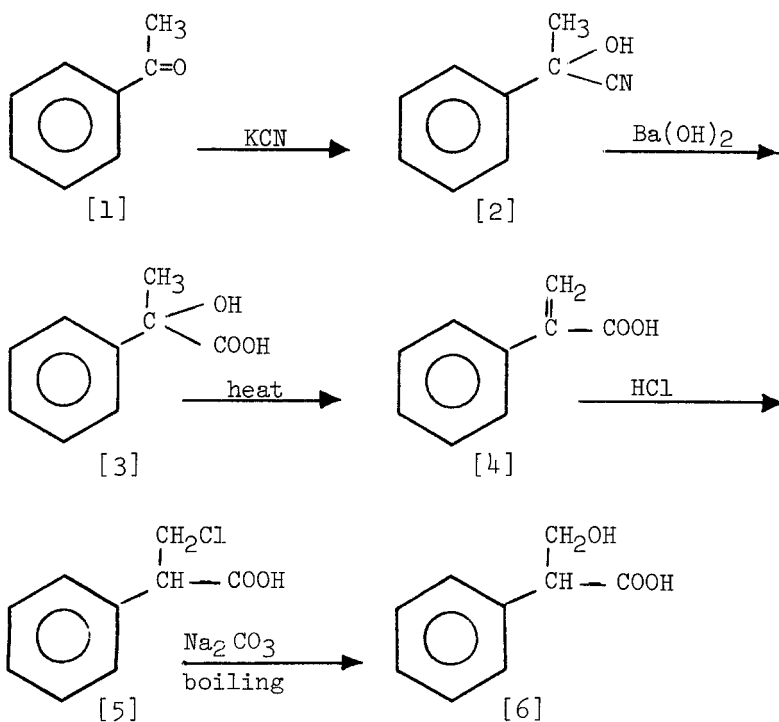
Scheme IV: Chambon's synthesis (33).

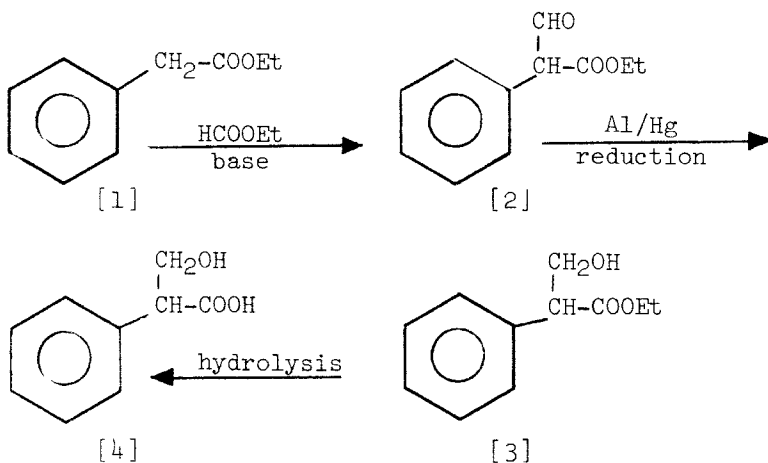
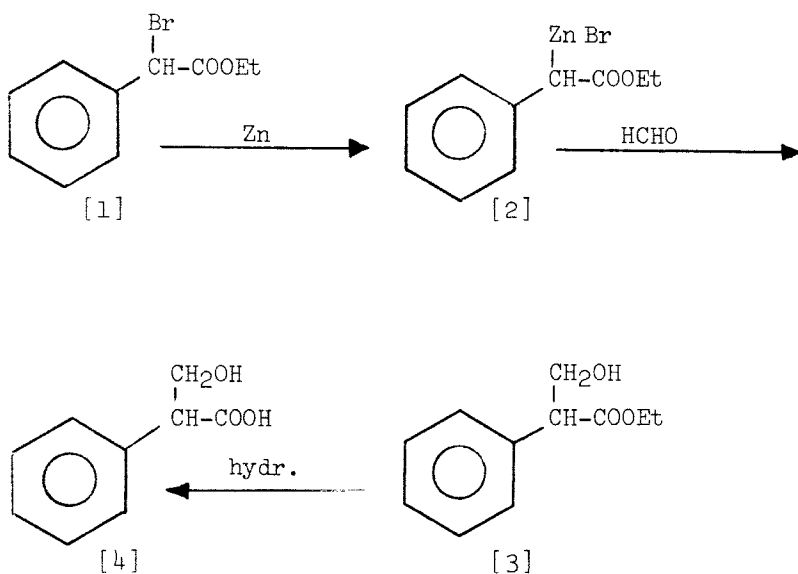
Ethyl  $\alpha$ -bromophenylacetate [1] is treated with Zn to give ethyl- $\alpha$ -zincbromophenylacetate [2] which is treated with formic acid to give dl-tropic ester [3] which upon hydrolysis yields tropic acid [4].

Scheme I: Landenburg's synthesis



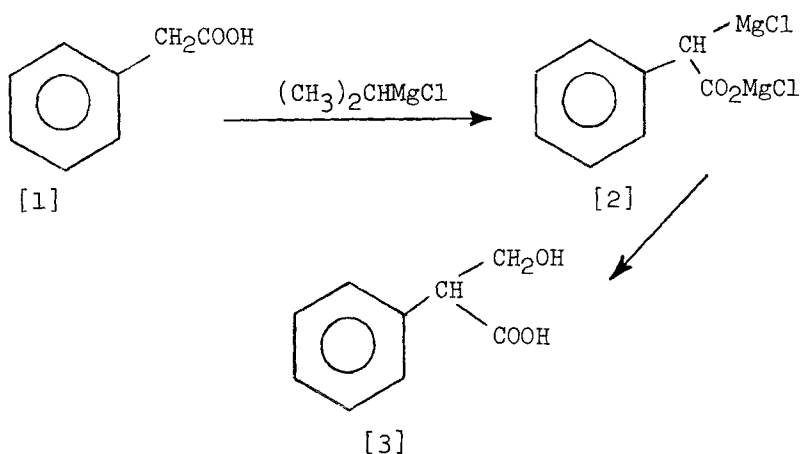
Scheme II: McKenzie and Wood's synthesis



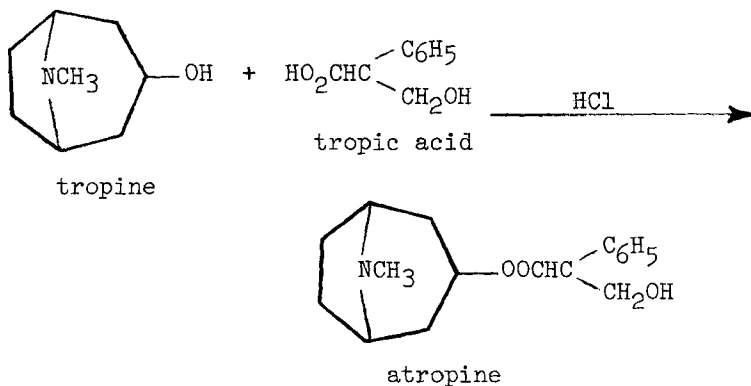
Scheme III: Müller's synthesisScheme IV: Chambon's synthesis

Scheme V: Blicke's synthesis (34).

Phenylacetic acid [1] is boiled with isopropylmagnesium chloride in ethereal solution to give [2] and then treated the product [2], a Grignard reagent with formaldehyde to give tropic acid [3].

Scheme V: Blicke's synthesis

Tropine finally can be combined with tropic acid to give atropine. This can be done by heating the two together in the presence of hydrogen chloride (Fischer-Speier esterification).



### 4.2.3 Synthesis of Labeled Atropine

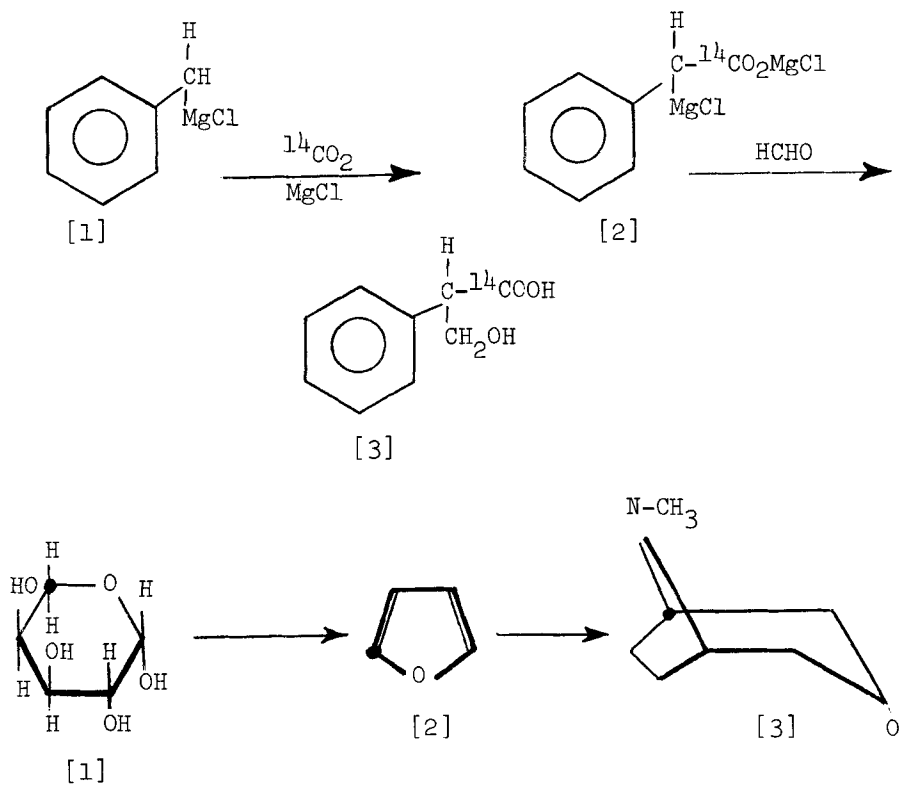
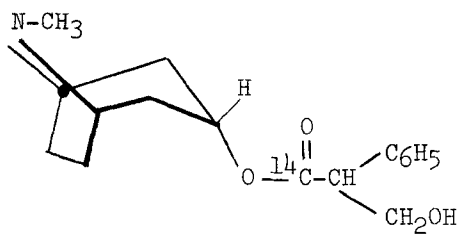
#### 4.2.3.1 Synthesis of Labeled Tropic acid

Benzylmagnesium chloride [1] is treated with  $^{14}\text{C}\text{O}_2$  followed with magnesium chloride to give the condensate [2]. This upon the addition of formaldehyde gives labeled tropic acid [3]. Synthesis of labeled tropic acid is presented in scheme VI (35).

#### 4.2.3.2 Synthesis of Labeled Tropine

- Synthesis of tropine-6, 7 T has been achieved by catalytic tritium addition to 2, 5-dimethoxy-2, 5 dihydrofuran and following Robinson's route to tropinone-6, 7 T, by subsequent reduction with hydrogen over Raney nickel (36).
- Synthesis of methyl- $^{14}\text{C}$  labeled tropine is carried out from Na  $^{14}\text{CN}$  (37) via methylamine- $^{14}\text{C}$  and based on Robinson's route; methyl- $^{14}\text{C}$  tropinone is obtained in 70% overall yield and tropine- $^{14}\text{C}$  in 68% yield.
- Synthesis of 1- $^{14}\text{C}$  tropine can be started with arabinose-5- $^{14}\text{C}$  [1] conversion into furan [2] and application of the Clauson-Kaas route to succinaldehyde and then to 1-or 5- $^{14}\text{C}$ -tropinone [3] (38). Using arabinose-3, 4- $^{14}\text{C}$  gives 6, 7- $^{14}\text{C}$ -tropinone (39) Scheme VII.

- 4.2.3.3 Labeled atropine can be then obtained by esterification of labeled tropic acid or labeled tropine to give either labeled atropine or double labeled  $^{14}\text{C}$  atropine (arised from labeled tropic acid and labeled tropine).

Scheme VI: Synthesis of Labeled Tropic acidScheme VII: Labeled tropine

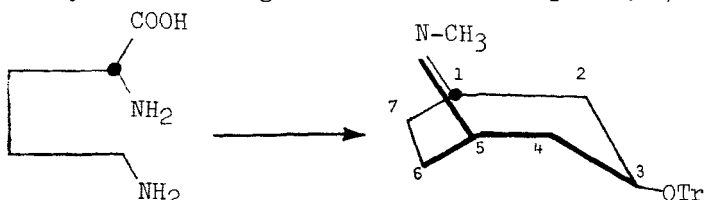
Double labeled atropine

## 5. Biosynthesis of Atropine

Most studies on the biosynthesis of atropine and of its isomer hyoscyamine have been performed on various species of *Datura*, but all the available evidence suggests that similar pathways occur in other tropane alkaloid-producing plants (26). Because the characteristic alkaloids of the group are esters of hydroxylamines and various acids (tropic, tiglic, etc.) there are, for each alkaloid, two distinct biosynthetic routes (26).

### 5.1 Biosynthesis of tropine

Ornithine and the related aminoacids (glutamic acid, proline) have been proved to be the precursors of the pyrrolidine ring of tropine (40-45). It was found that feeding [2-<sup>14</sup>C] ornithine to *Datura stramonium* resulted in radioactive hyoscyamine labelled only at C-1 bridgehead carbon of tropine (46).



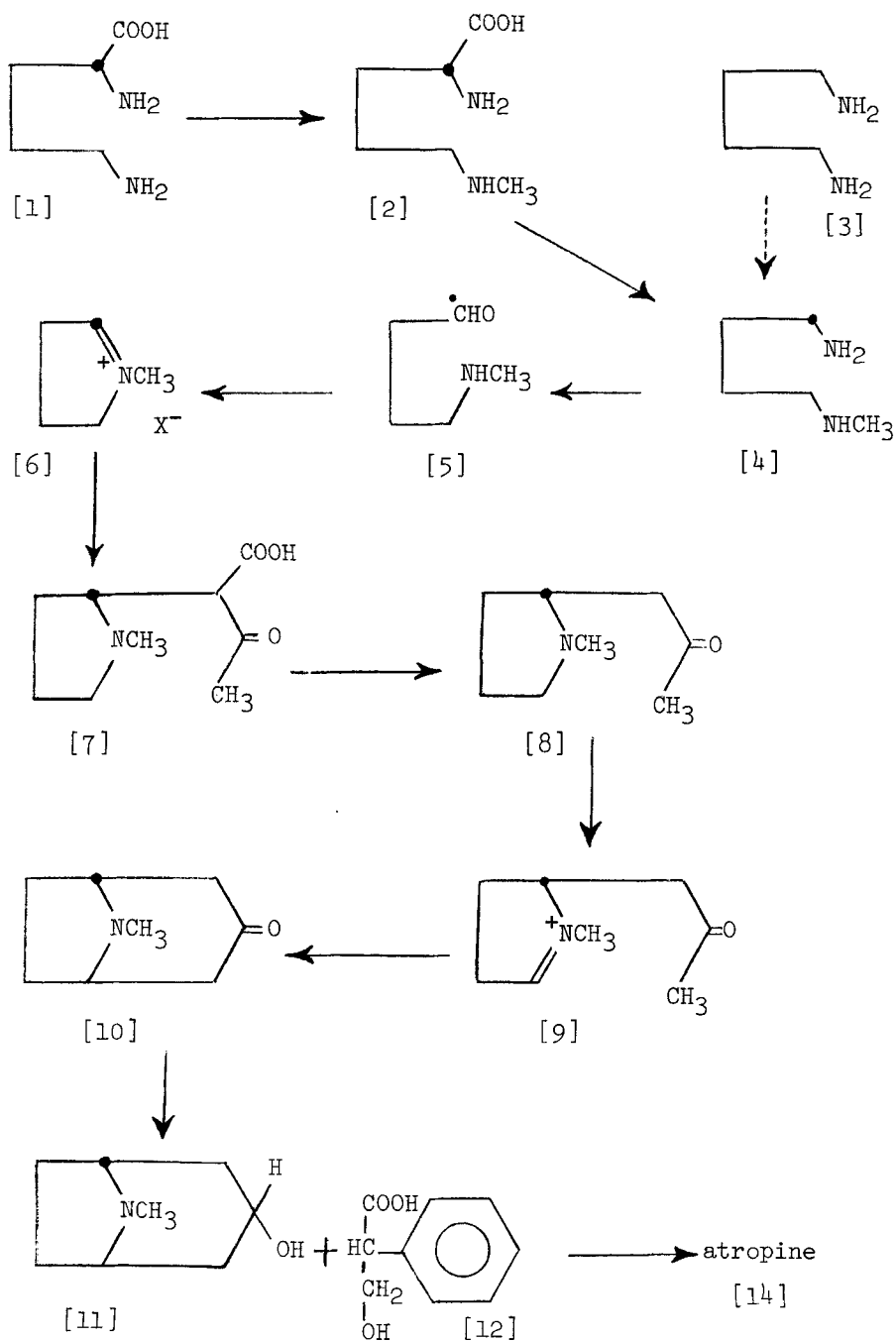
And that [5-<sup>14</sup>C] proline resulted in radioactive hyoscyamine labelled only the C-5 position of tropine (44).

It was also reported that [2-<sup>14</sup>C,  $\delta$ -<sup>15</sup>N] ornithine incorporated into tropine moiety of hyoscyamine and the  $\delta$ -aminogroup of ornithine is an efficient precursor of the tropine nitrogen (44,46).

The incorporation of glutamic acid and proline is considered to occur via ornithine (46).

Ornithine [1] is incorporated into tropine via  $\delta$ -N-methylornithine [2] (47-49) as [methyl-<sup>14</sup>C]- $\delta$ -N-methyl-[2-<sup>14</sup>C] ornithine was incorporated into hyoscyamine labelling C-1 and the N-methyl group. [2] is decarboxylated to yield N-methylputrescine [4] (50,51).

Putrescine [3] has also been shown to be a precursor of the tropane alkaloids (43,52-54). It was suggested (46) that putrescine [3] is converted by certain enzymes in *Datura* plants to N-methyl putrescine [4]. Oxidation of the primary alcohol of [4] affords 4-methylaminobutanal [5]. This is cyclized to give N-methyl- $\Delta^1$ -pyrrolinium salt [6].

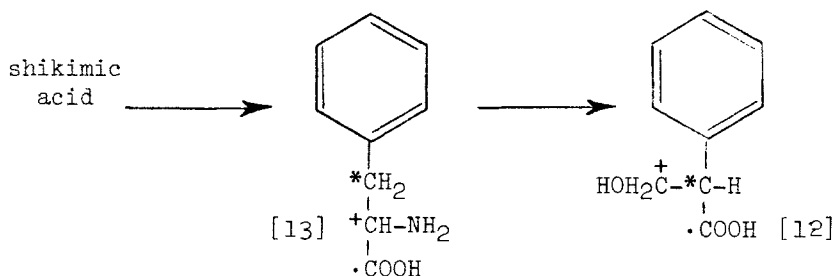
Leete's Scheme: Biosynthesis of Atropine



Carbons 2, 3 and 4 of tropine are derived from acetate (55,56) and it is assumed that the acetate is incorporated via acetoacetic acid or some suitable activated derivative such as coenzyme A ester (46). [6] is therefore condensed with acetoacetate to give hygrine- $\alpha$ -carboxylic acid [7]. Decarboxylation of [7] affords hygrine [8] which is an established precursor of tropine (56,57). [8] is dehydrogenated to give dehydrohygrine [9]. The latter is cyclized to yield tropinone [10]. Stereospecific reduction of [10] affords tropine [11].

## 5.2 Biosynthesis of tropic acid

Tropic acid [12] is formed by the intramolecular rearrangement of phenylalanine [13] (58). Compounds which are metabolically related to phenylalanine such as phenylpyruvic acid are also incorporated into tropic acid (59,60).



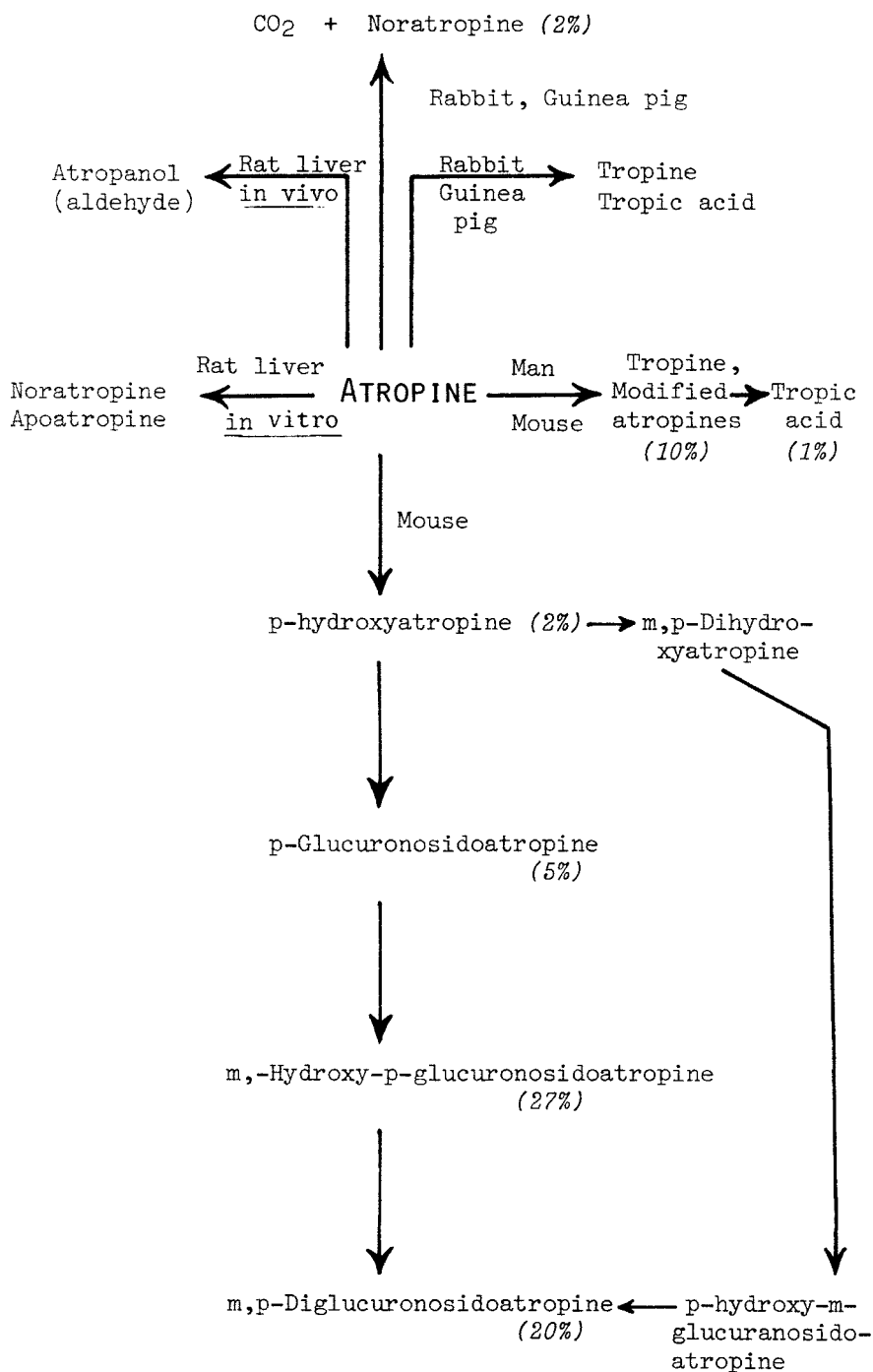
Tropine [11] is finally esterified with tropic acid [12] to give atropine [14].

## 6. Metabolism of Atropine

Atropine is rapidly absorbed from the gastrointestinal tract and readily absorbed from the mucous membranes and the skin (21, 61). Absorption from the intestinal tract is complete in 2 hours. About one-half of the atropine circulates in the free form in the blood and the other half is bound by the plasma proteins (21). Atropine also enters the circulation when applied locally to mucosal surfaces of the body (61). The transconjunctival absorption of atropine is considerable. About 95% of radioactive atropine is absorbed and excreted following subconjunctival injection in the rabbit (62). The metabolism of atropine varies considerably from one species to another. Hydrolysis to tropine and tropic acid is not thought to be a major metabolic route since only traces of tropic acid are recovered in the urine (21).

Atropine disappears rapidly from the blood and is distributed throughout the entire body (21). The liver, kidney, lung and pancreas are the most important organs that take up the labeled atropine (62). Most is excreted in the urine within the first 12 hours, in part unchanged (21). Following intra-muscular administration of a single 2 mg doses of  $^{14}\text{C}$ -labelled atropine in man, Gosselin et al. (63) found that 85 to 88% of the radioactivity was excreted in the urine within 24 hours, only a trace could be extracted from the faeces; about 50% of the dose appeared in the urine unchanged, over 30% was excreted as unknown metabolites and less than 2% appeared as free tropic acid. After intravenous injection of atropine in the mouse, approximately 25% of the dose is excreted in the urine as atropine, more than 50% as conjugates with glucuronic acid and the remaining 20-25% as intermediate oxidation products (probably p-hydroxyatropine and 3,4-dihydroxyatropine) and tropine-modified atropines (62). The metabolism of atropine is presented in scheme I [after (62)].

## SCHEME I: THE METABOLISM OF ATROPINE



## 7. Pharmacokinetics

The pharmacokinetics of atropine were reported by several authors.

Peak serum levels occur approximately 30 minutes following intramuscular (I.M.) administration of 1 mg dose of atropine (64).

Serum levels following intravenous (I.V.) administration of atropine drop within the first 10 minutes and then decrease more gradually. Levels one hour following either I.V. or I.M. administrations are very similar (64).

Following I.M. administration of 2 mg atropine, the onset and duration of effect on heart rate are reported (65) to be maximum at 15-50 minutes and up to 5 hours, respectively.

Following endotracheal administration of 1 mg atropine sulfate, serum levels of atropine were less than 5 $\mu$ g/ml at 30 seconds and 11 $\mu$ g/ml at 10 minutes (66).

Atropine's half-life is reported to occur at two rates, with an initial fast rate of about 2 hours and a slow rate ranges 12.5-38 hours (65).

The average half-life of atropine is 4.125 hours following a single 1 mg intravenous dose of atropine in humans (67).

The mean total plasma clearance of six normal human volunteers following a single 1 mg intravenous dose of atropine is reported to be 533.35 ml/minute (67).

Maximum cycloplegia usually occurs within several hours of administration of topical atropine, though effective cycloplegia may occur in 30 to 40 minutes (68).

The mydriatic effect may persist for up to 10 days while the cycloplegic action may last for 5 days (68).

8. Therapeutic Uses of Atropine (69)

1. Pre-anaesthetic medication:
  - to decrease secretions of salivary, naso-pharyngeal and bronchial glands.
  - to prevent reflex broncho-spasm.
  - to reduce reflex bradycardia of inhalational anaesthetics.
2. Antispasmodic in:
  - Bronchial asthma.
  - Renal, biliary and intestinal colic.
  - Peptic ulcer.
  - With purgatives.
3. Vaso-Vagal syncope due to reflex lowering of blood pressure and severe Bradycardia.
4. Nocturnal enuresis and urgency of micturition to decrease urinary bladder reflex irritability.
5. In Parkinsonian disease to reduce rigidity (central action).
6. Antidote for parasympathomimetic poisoning e.g. organo-phosphorous insecticide poisoning.
7. Mydriatic and cycloplegic in:
  - Iritis
  - Keratitis
  - Corneal ulcerations or injuries

## 9. Methods of Analysis

### 9.1 Identification Tests

The following identification tests are mentioned in the British Pharmacopoeia of 1963 (70)

- 1 mg of atropine is added to 4 drops of fuming nitric acid and the mixture is evaporated to dryness on a water bath; a yellow residue is obtained. 2 ml of acetone and 4 drops of a 3% w/v solution of potassium hydroxide in methyl alcohol are added to the cooled residue; a deep violet color is produced.
- 50 mg of atropine is dissolved in 5 ml of water acidified with hydrochloric acid, gold chloride solution is added; a lemon-yellow oily precipitate is formed which rapidly crystallizes. This precipitate after recrystallization from boiling water acidified with hydrochloric acid, has a minutely crystalline character, is dull and pulverulent when dry, and has a melting point about 136°.

Other identification tests are as follows:-

- The Gerrard reaction (71).  
To about 6 mg of atropine, 1 ml of 2% solution of mercuric chloride in 50% aqueous methanol is added; a deep red color is produced.
- To a trace of atropine in an evaporating dish, drops of the p-dimethylaminobenzaldehyde reagent (2 g of p-dimethylaminobenzaldehyde is dissolved in 6 gm sulfuric acid) are added as well as 0.4 ml of water. The resulting mixture is heated on a boiling water bath; an intense red color is produced which changing to permanent cherry red on cooling.
- Physiological test: Induction of mydriasis (can be performed on young cats, dogs and rabbits).  
An aqueous, alcohol free solution of atropine or its sulfate is dropped into the conjunctival sac of the eye and held so that none is lost by overflow of tears. It has been reported (71) that 1 part in 40,000 or that 0.000,000,427 g of atropine sulfate will cause a distinct dilation of the pupil of the eye in 1 hour.

## 9.2 Microcrystal tests

100 mg of atropine dissolved in 5 ml water acidified with dilute sulfuric acid. The following microcrystals were performed.

- Picric acid with atropine gives bunches of plates (21). The crystals are shown in Fig. 7.
- Wagner's reagent with atropine gives irregular hexagons in clusters (21). The shape of crystals is shown in Fig. 8.
- Dragendorff's reagent with atropine gives irregular rectangles as shown in Fig. 9.
- Mercuric chloride with atropine gives long prisms as shown in Fig. 10.

## 9.3 Titrimetric Methods

### 9.3.1 Aqueous Titrations

Bobtelsky and Barzily (72) have reported a misoheterometric titration of large, organic, nitrogen-containing compounds including atropine. Micro amount of atropine is titrated heterometrically with tungstosilicic acid, tungstophosphoric acid or molybdophosphoric at pH 1 or 7.

Other titrimetric methods for the assay of atropine have been published:

- a) Determination of atropine, tropine and tropic acid in decomposed atropine products (73).
- b) The application of sodium dodecyl sulfate titrimetric solution in the analysis of atropine injections (74).
- c) The influence of salts, polyhydric compounds and absorbents on the determination of organic bases by anionic surfactant in two-phase systems. The method was applied to atropine among other organic bases (75).
- d) Atropine in aerosol has been determined titrimetrically by slowly ejecting the sample (2 g) through a standard solution of acid and titrating the excess acid (76).

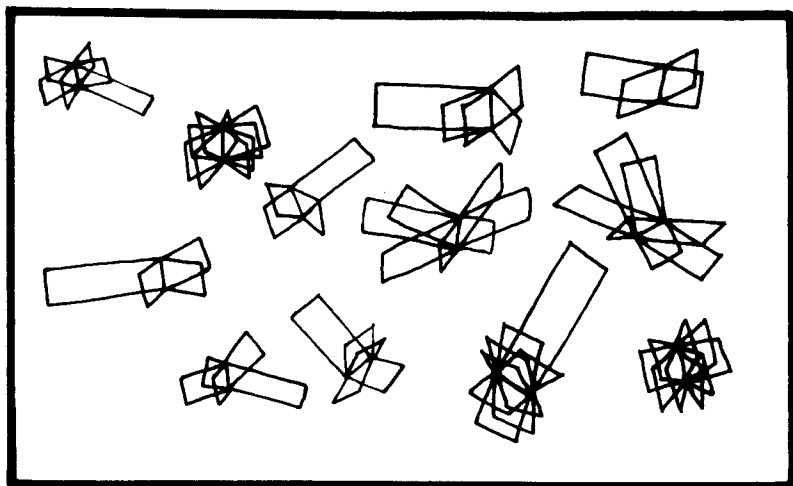


FIG. 7. MICROCRYSTALS OF ATROPINE WITH  
PICRIC ACID.

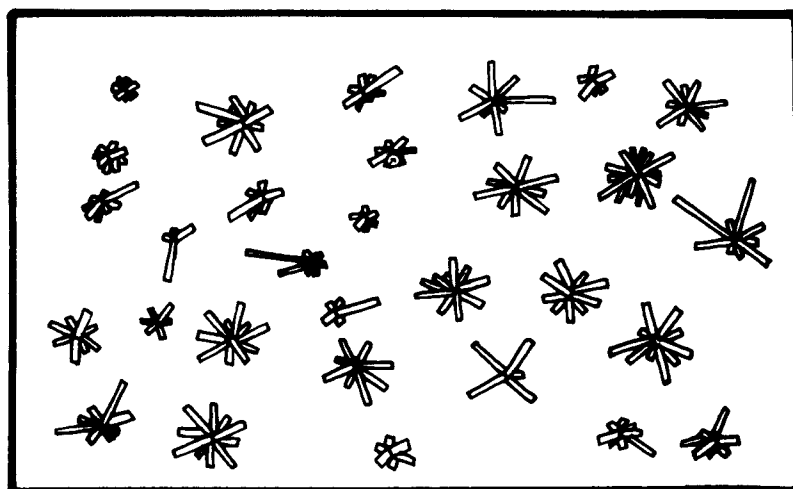


FIG. 8. MICROCRYSTALS OF ATROPINE  
WAGNER'S REAGENT.



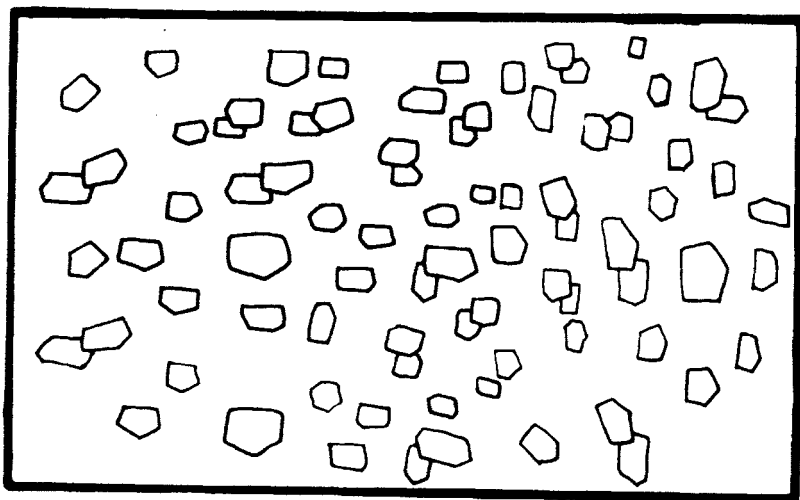


FIG. 9. MICROCRYSTALS OF ATROPINE  
DRAGENDORFF'S REAGENT.

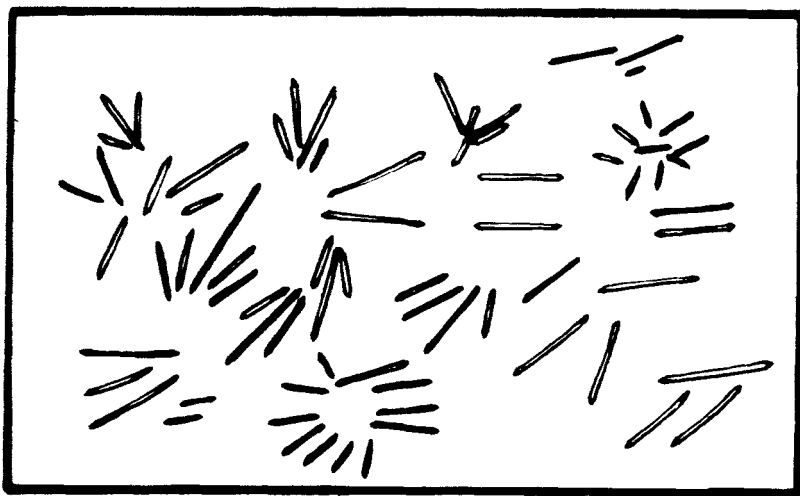


FIG. 10. MICROCRYSTALS OF ATROPINE  
WITH MERCURIC CHLORIDE.

- e) The influence of atropine among other organic bases on the partition of indicator acids in a water-chloroform system (77).
- f) Atropine was detected and quantitatively determined in decomposing tissues (78).

A direct titration method using lead nitrate was described for drug products including atropine sulfate (79).

### 9.3.2 Non-Aqueous Titration

The USP XX 1980 (80) described a non-aqueous titration for the assay of atropine as follows:

Dissolve about 400 mg of atropine, accurately weighed, in 50 ml of glacial acetic acid, add 1 drop of crystal violet TS, and titrate with 0.1 N perchloric acid VS to a green end-point. Perform a blank determination and make any necessary correction.

Each ml of 0.1 N perchloric acid is equivalent to 28.94 mg of atropine ( $C_{17}H_{23}NO_3$ ).

The British Pharmacopoeia 1980 (81) describes a non-aqueous titration for the assay of atropine as follows:

Dissolve 0.3 g in 20 ml of anhydrous glacial acetic acid, and titrate with 0.1 M perchloric acid VS and determine the end-point potentiometrically.

Dzyuba and Shraiber (82) have quantitatively determined atropine by titration in non-aqueous solvents. The total alkaloids of the atropine group (atropine plus hyoscyamine plus hyoscyne) are determined by titration against  $HClO_4$  in anhydrous acetic acid. The method is applied to leaves, extract and tincture of belladonna, and to tablets, suppositories and eye-drops containing atropine or belladonna. The end-point is determined potentiometrically (quinhydrone electrode with S.C.E. as comparison electrode) or with crystal violet as indicator.

Symoni and Tokar (83) have reported new reagent for titrations of atropine and other alkaloids in non-aqueous media by means of the hydrochloric acid

complex of aluminium isopropoxylate. The standard solution containing the HCl complex of aluminium chloroisopropoxylate is prepared by dissolving aluminium chloroisopropoxylate in chloroform and passing the calculated amount of HCl gas into it, or by adding the stoichiometric amount of chloroform solution of aluminium chloroisopropoxylate to a standardized solution of HCl (3% to 4%) in dry chloroform. The solution must be kept very dry.

The above authors (84) have also reported a new reagent for titrations in non-aqueous media. The determination of atropine and other alkaloids by means of the hydrochloric acid complex of chloro aluminium isopropoxide. Results were discussed which have been shown that the HCl complex of chloro aluminium isopropoxide behaved as a monobasic acid when undergoing salt formation with various alkaloids. The author have given a method for the determination of atropine (and other alkaloids) with 0.1 N chloro aluminium isopropoxide in chloroform. The deviation was  $\pm 1\%$  in the range 38 to 245 mg of alkaloid.

Simon et al (85) have described a method for the determination of trace amounts of atropine by titration in anhydrous solvents. For solid atropine sulfate, dissolve the sample in anhydrous acetic acid, add 0.1% p-dimethyl aminoazobenzene solution in benzene, and titrate with 0.005 N -  $\text{HClO}_4$  until the color changes from yellow to pink. For aqueous solution of atropine sulphate, make alkaline with aqueous sodium bicarbonate, extract with chloroform and titrate the extract as described above.

### 9.3.3 Gravimetric Titration

Poethke and Trabert (86) have utilized potassium iodobismuthate for the determination of small quantities of atropine and other alkaloids. The method is based on the principles developed for the determination of 8-hydroxyquinoline (87) is described. The drug is determined by precipitating its iodobismuthate and, either determining it gravimetrically.

The above authors (88) have also determined atropine in ampules, eye ointment, pills and extracts of belladonna, and in tablets and stomach powders containing belladonna. Good results were obtained when

assaying comparatively small amounts of the drug.

Van Pinxteren et al (89) have reported the determination of atropine by means of tetraphenylboron (Kalignost). By using Flaschkas sodium tetraphenylboron method (90,91) for the determination of atropine in alkaloidal salts and galenicals, recoveries varying from 81.9 to 99.6% were obtained according to the volume of solution analysed. Reasonable results were obtained by reducing the volume of solution to 25 ml and with 10 to 25 mg of atropine. By applying the gravimetric method to 50 to 100 ml samples of *Maceratum Radicis Belladonnae*, accurate results were obtained over the range of about 0.020 to 0.035% of atropine.

#### 9.3.4 Potentiometric Titration

Pernarowski and Blackburn (92) have carried out a potentiometric titration of atropine. The titration is carried out in chlorobenzene with glass and sleeve-type calomel electrodes; 0.05 N  $\text{HClO}_4$  in glacial acetic acid is the most suitable titrant. Bromophenol blue is a suitable indicator for titrations in chlorobenzene to a visual end point. The results of six titrations of atropine showed an average recovery of 99.7% and standard deviation of 0.55%.

#### 9.4 Polarographic Methods

Souckova and Zyka (93,94) have reported two polarographic titration methods for titration of organic bases including atropine. The first method is the titration with tungstosilicic acid, and the second is titration with tungstophosphoric and molybdo phosphoric acids. The latter method is reported to be unsatisfactory for atropine. The first method allows accurate determination of 10 to 20 mg of a base.

Novotny (95) have published a polarographic determination of atropine in mixtures. The drug is extracted from alkaline solution with chloroform, evaporated and atropine is nitrated with  $\text{HNO}_3\text{-H}_2\text{SO}_4$  mixture (> 10:1) on water bath for 30 minutes. The mixture is made alkaline and, after removing oxygen by means of nitrogen, polarography of the solution is carried out. The polarogram is compared with one prepared from a similar sample to which a known amount of atropine is added.

An Osillopolarographic study of atropine and other alkaloids is reported by Habersberger and Zyka (96). Osillopolarographic curve of atropine was studied with a dropping mercury electrode. A carbon electrode was used a reference electrode.

Some aspects of the polarographic determination of atropine is reported by Benraad and Uffellie (97). Experimental evidence is produced which indicates the reaction of atropine at the dropping mercury electrode in 0.1 N LiCl is a simple reduction process.

## 9.5 Spectrophotometric Methods

### 9.5.1 Colorimetry

Atropine has been determined colorimetrically, among other atropa alkaloids, by the use of new reagents. An absorptiometric method is described (98) for the determination of atropine and related alkaloids. The well-known Vitali-Morin reaction was investigated with a view to improving the stability of the colored formed. It was found that the best results were obtained with tetraethylammonium hydroxide as the base and dimethylformamide as the solvent. The solution (0.05-0.15 mg of alkaloid) is evaporated to dryness, nitrated with 0.2 to 0.3 ml of fuming  $\text{HNO}_3$ , again evaporated, dissolved in dimethylformamide, treated with 0.3 ml of 25 percent aq. tetraethylammonium hydroxide and diluted to 10 ml with dimethylformamide. The optical density is determined at 540 m $\mu$  in 1-cm cells against dimethylformamide and the alkaloidal content is ascertained from a calibration graph which is linear.

Simonyi and Tokar (99) have reported a new colorimetric method for the determination of small amounts of tropic acid and its esters. Atropine was nitrated for 15 minutes at 50° with a solution of 20%  $\text{KNO}_3$  in conc.  $\text{H}_2\text{SO}_4$ . On making the product alkaline with hot 18 to 20% NaOH, a color develops in 30 minutes. This is estimated by using an S42, S47 or S50 filter in the Pulfrich photometer. The sensitivity is 50 and 60  $\mu\text{g}$  of atropine per ml. The probable error is  $\pm 3\%$ .

Nir-Grosfeld and Weissenberg (100) have reported two colorimetric methods for the determination of atropine in pharmaceutical preparations. Recovery experiments indicate an accuracy of  $\pm 1\%$ . The results agree with these obtained by the method of USP XV.

In method I, a chloroform extract, prepared by the USP method, is evaporated to dryness on a water bath. Nitric acid (fuming) was added, and heated till fuming ceased, dried at  $105^\circ$  for 15 min and allowed to cool. The residue obtained was dissolved in acetone and diluted to 25 ml. An aliquot (5 ml) was mixed with isopropylamine and 0.1% methanolic KOH and the extinction at 540 mu was measured after one minute.

In method II. The compound is nitrated as in method I and dissolved in 50% ethanol (10 ml). Heated on a water bath with 1% HCl and zinc dust for 10 minutes, cooled and filtered. The zinc residue was washed with  $H_2O$  and the washings were added to the filtrate. 1% of  $NaNO_2$  is added, mixed and allowed to stand for 10 minutes. To this 92.5% solution of ammonium sulphamate was added, shaken and allowed to stand for 10 minutes. N-1-naphthylethylenediamine dihydrochloride solution was added, diluted with water to 25 ml and after 30 min, the extinction at 550 mu was measured.

Pohm (101) reported a micro-determination of atropine colorimetrically, by means of p-dimethylaminobenzaldehyde. Atropine is mixed with ether and aq.  $NH_3$  and sit aside for two hours and filtered. The filtered extract is extracted with 0.05 N HCl. The HCl extract is made alkaline (NaOH) and extracted with chloroform, evaporated to dryness. Three drops of aq. bromine are added and evaporated off. The residue is dissolved in methanol and again evaporated with 3 drops of aq. bromine. After drying for 2 hours over  $P_2O_5$ , the residue is treated with 7 drops of Wasicky reagent (a solution of 1 gm of p-dimethylaminobenzaldehyde in 9 gm of 88%  $H_2SO_4$ ) and sit aside for 2 minutes. It is then heated for 3 minutes in

a boiling water bath and cooled in ice for 15 seconds. Acetic anhydride is added with stirring and after 30 minutes, the extinction is measured at 500 mu.

Atropine has been determined (102) colorimetrically by means of Reineck's salt. Ammonium reineckate was used for the determination of atropine in 1%  $H_2SO_4$ . Ammonium reineckate solution (0.5%) was added to the test solution, the mixture was placed in a refrigerator for 30 minutes and the precipitate is collected on a glass filter, washed with cooled water and dissolved in acetone. The extinction is then measured against a reagent blank.

The extraction-spectrophotometric determination method for the assay of atropine with the use of vanadium catecholate has been reported by Shesterova et al. (103). The method involves formation of a water-insoluble V'V-catechol-atropine (1:2:1) complex (I) in an aq. medium adjusted to pH 3 to 4 with hydrogen phthalate buffer solution containing a 200-fold molar excess (relative to I) of  $VO_3^-$  and an 8000-fold molar excess of catechol and extraction of this complex into chloroform. The complex exhibits max. absorption at 620 nm.

Semenicheva (104) reported a method for the determination of atropine sulphate in eye drops. Atropine sulphate in neutral solution is treated with sodium picrate and the atropine picrate formed is extracted with chloroform; after removal of chloroform, the picrate is treated with sodium sulphide solution and the color of the sodium picramate formed is compared with standard prepared by reducing picric acid solution in the same way.

#### 9.5.2 Photometric Analysis

Akopyan (105) has reported a photometric method for the determination of atropine and other tropan alkaloids in pharmaceutical mixtures. The determination is based on the reaction of the alkaloid (atropine) with p-aminobenzaldehyde on concentrated sulphuric acid. The

intensity of the color produced being measured in a photometric absorptiometer with a green filter.

Fahmy et al. (106,107) have published a comparative study of the different photometric methods of determination of atropine:-

- I. The tungstosilicic acid, tungstophosphoric acid, copper sulphate, sodium picrate and p-dimethylaminobenzaldehyde methods are suitable for the microdetermination of atropine in toxicological samples. Vitali's method is preferred.
- II. The use of bromothymol blue, bromocresol purple, Metanil yellow (C.I. acid yellow 36) and methyl orange, and various organic solvents, in the alkaloid-dye method of determination has been studied. The combination of Metanil yellow and chloroform is most convenient.

The use of ammonium reineckate in the photometric determination of atropine, has been described (108). The procedure is as follows:

To the solution containing from 2 to 10 mg of atropine add 0.5 N  $\text{H}_2\text{SO}_4$  (2 drops) and saturated ammonium reineckate solution, with stirring. Collect the precipitate on a sintered-glass filter ( $G_4$ ), wash it with cold water, and dissolve it in dioxan acidified with 0.5 N- $\text{H}_2\text{SO}_4$ . Measure the extinction of the dioxan solution at 530 m $\mu$ , and refer the results to a calibration curve. The method was used for determining atropine in tablets.

Levine and Roe (109) have described a method for the determination of atropine and tropic acid.

Atropine and tropic acid were separated from each other and from preservatives such as benzyl alcohol or phenol by partition chromatography and determined by a modified Vitali procedure. The chromatographic procedure employs two columns connected in series, with Celite 545 as supporting phase. In column A the



sample (2 ml) made alkaline with N  $\text{NaHCO}_3$  (1 ml) absorbed on Celite (4 g + 1 g), constitutes the stationary phase and in column B the stationary phase is 0.2 N  $\text{H}_2\text{SO}_4$  (2 ml) absorbed on Celite (3 g + 1 g). On washing the columns with chloroform (100 ml + 25 ml through column B only), tropic acid remains on column A. Atropine is absorbed column B, and preservatives pass both columns. Tropic acid is eluted from column A with either after acidification of the column with acetic acid in ether, and atropine is eluted from column B with chloroform after neutralization of the column with aqueous ammonia. Atropine and tropic acid are converted into their salts by addition of HCl and aqueous ammonia respectively, and evaporated to dryness. For the modified colorimetric procedure, treat the dry residue on a steam bath for 30 minutes with fuming nitric acid (1 ml) in a covered flask. Add water (10 ml), aqueous ammonia, (2 ml), sodium dithionite (about 50 mg) and 5%  $\text{NaNO}_2$  solution (5 ml) and heat for a further five minutes, add 5% sulphamic acid solution (10 ml) and remove nitrous fumes in a current of air. Add 25 mg of solid N-1-naphthylethylenediamine dihydrochloride, make up to volume, set aside for 0.5 to 4 hours, and compare the extinction at 550 mu with values obtained from standards treated similarly. Beer's law is obeyed up to at least 4 mg recoveries were from 100 to 103%.

Febvre (110) reported that Vitali-Morin reaction for atropine is modified to give a reproducible color that can be used quantitatively. A known volume of the sample is evaporated to dryness under vacuum in a centrifuge tube, then treated with a few drops of a mixture of 7 ml of  $\text{H}_2\text{SO}_4$  (66° Be') and 2 ml of fuming  $\text{HNO}_3$  and stirred to make the solution homogenous. Acetone (2 ml) is added and 10% absolute ethanolic KOH (the presence of water or methanol vitiates reaction) drop by drop until the solution is neutralized, when the color appears at once. After centrifugation to remove solid precipitated by the acetone and making up to 10 ml with acetone; the extinction is measured (Filter 63 of the Jobin - Yvon Spectrophotometer).

The extinction is stable for 10 minutes at 20°. The Beer-Lambert's law is followed only for concentration from 5 to 20 µg per ml, but for higher concentration, a calibration curve can be used. Above 100 µg per ml the sensitivity falls off. The mean error is about 1%. No color is given by the hydrolysis products of atropine.

### 9.5.3 Ultraviolet Spectrophotometric Methods

Systematic toxicological analysis by spectrophotometric methods have been published (111). The sample of tissue is homogenized with 25 ml of 0.1 N HCl; the homogenate is extracted on a water bath with 75 ml 95% ethanol and 2 ml, 10% Na<sub>2</sub>WO<sub>4</sub>. The residue is being dissolved in 50 ml of McIlrains's buffer at pH 7 and extracted with chloroform (50 ml). The separated chloroform layer is then extracted 100 ml of 0.1 N HCl. The characteristic U.V. absorption curves for 30 alkaloids in dil. HCl are presented; atropine can be determined quantitatively by this method.

Cross *et al.* (112) have determined some alkaloids including atropine spectrophotometrically and described its application to pharmaceutical preparations. To determine atropine, add 1% sodium picrate solution (3 ml) to a solution of atropine (1 mg) in phosphate buffer solution (pH 7) (20 ml), extract with chloroform, shake the extract with phosphate buffer solution, (pH 11.2 to 11.5) (40 ml) dilute the aq. phase with the same buffer solution to 100 ml, and measure the extinction at 355 mµ.

Waalder and Bjerkelund (113) have described the following procedure, for the ultraviolet determination of apotatropine and belladonine in atropine:

"Prepare a solution of the mixture in 0.1 N - H<sub>2</sub>SO<sub>4</sub> containing 15% of ethanol, and measure the extinction at 261.5, 257.5 and either 248.5 or 254.0 mµ". Calculate the content of each alkaloid by solution of the three appropriate simultaneous equations. The extinction coefficient of each compound at each wavelength is given.

Atropine was determined spectrophotometrically in eye drops by Zabrack and Farkas (114). The absorption spectra of atropine show a maxima at 186 m $\mu$ . Dilute 1 ml of the sample to 100 ml and 5 ml of this solution is further diluted to 100 ml with water and measure the extinction at 186 m $\mu$  against water. Beer's law is obeyed over the range 0 to 8  $\mu$ g per ml. The results obtained by this method are within 1% of those obtained by extraction methods.

Uhlmann (115) reported a spectrophotometric assay method for atropine and some narcotics and alkaloids in galenical compositions. To assay the drug in aq. solution of its salt, the extinction of the diluted sample is determined at the wavelength for maximum absorption (257 to 286 nm) and compared with that of progressively diluted samples of stock solution. The method is chiefly designed for use on aq. preparations (ampoules).

#### 9.5.4 Infra-red Spectrophotometric Method

The application of infra-red spectrometry to quantitative analysis of atropine in the solid phase has been reported by Browning et al. (116). The pressed potassium bromide pellet technique has been successfully applied as an aid in the quantitative determination of atropine by IR spectrophotometry.

#### 9.5.5 Fluorometric Analysis

Laugel (117) have published a method for the determination of atropine and, other alkaloid, based on the fluorescence of compounds of the type acid dye-azo base. The concentration of atropine in pharmaceutical preparation is determined (to within 4%) by measuring the fluorescence of the complex formed quantitatively, in chloroform solution, by atropine with a dihydroxylluran acid dye, e.g. cosin. The concentration which is directly proportional to the fluorescence (measured at 550 m $\mu$ ), is obtained from a standard calibration curve for atropine. Beer's law being obeyed for 10 to 60  $\mu$ g of atropine.

Shuntaro Ogawa *et al.* (118) have determined the fluorimetry of atropine with eosin yellowish (C.I. Acid Red 87). The method which is simple and rapid is based on the formation of fluorescent complex between atropine and eosine. To a solution of atropine in chloroform (9 ml) is added 0.1% eosine solution (1 ml), the mixture is shaken thoroughly and the fluorescence intensity at 556 mμ (excitation at 365 mμ) is measured after 10 minutes. Beer's law is obeyed with 1 to 5 μg of atropine per ml; the coefficient of variation is 2.6%.

#### 9.5.6 Phosphorimetric Analysis

Winefordner and Tin (119) have determined atropine in urine. A rapid method was described for the extraction of atropine from body fluids; the concentration of the drug is determined by phosphorescence measurement and comparison with standard solution.

### 9.6 Chromatographic Methods

#### 9.6.1 Paper Chromatography

Clarke (21) described two systems:

- 1) Whatman No. 1, sheet 14 X 6 in, buffered by dipping in a 5% solution of sodium hydrogen citrate, blotting and drying at 25° for one hour. It can be stored indefinitely. A sample of 3 μl 1% solution in 2N acetic acid or in ethanol is used. Solvent system: 4.8 gm of citric acid in a mixture of 130 ml of water and 870 ml of n-butanol (this solvent may be used for several weeks if water is added from time to time to keep the specific gravity at 0.843 to 0.844). The chromatogram is developed, ascending in a tank 8 X 11 X 15½ in. 4 Sheets being run at a time. Location is done under ultraviolet light and the location reagent is iodoplatinate spray,  $R_f = 0.37$ .
- 2) Whatman No. 1 or No. 3, sheet 17 X 19 cm, impregnated by dipping in a 10% solution

of tributyrin in acetone and drying in air. A sample of 5  $\mu$ l of 1 to 5% solution in ethanol or chloroform, using acetate buffer (pH 4.58) as solvent. The beaker containing the solvent is equilibrated in a thermostatically controlled oven at 95° for 15 minutes. The chromatogram is developed, ascending, where the paper is folded into a cylinder and clipped. The cylinder is inserted in the beaker containing the solvent which is not removed from the oven. A plate-glass disk thickly smeared with silicone grease may serve as a cover. Time run 15 to 20 minutes. The location reagent is iodoplatinate spray and  $R_f = 0.94$ .

Other paper chromatography systems have been published (120-136).

#### 9.6.2 Thin-Layer Chromatography

Clarke (21) described the following system for the separation of atropine:

Glass plates 20 X 20 cm, coated with a slurry consisting of 30 g of silica gel G in 60 ml of water to give a layer 0.25 mm thick and dried at 110° for 1 hour. A sample of 1.0  $\mu$ l of 1% solution in 2N acetic acid, taken by a micro drop, is used. The solvent system consists of strong ammonia solution : methanol (1.5 : 100). It should be changed after two runs. Solvent is allowed to stand in the tank for 1 hour. The ascending chromatogram is developed in a tank 21 X 21 X 10 cm, the end of the tank being covered with filter paper to assist evaporation. Time of run 30 minutes. The location reagent is an acidified iodoplatinate spray: and the  $R_f$  value is 0.18.

Other TLC systems have been published (133-135, 137-140) for the separation of atropine.

#### 9.6.3 High Pressure Liquid Chromatography

Stutz and Sass (141) have described a high-speed, high pressure liquid chromatography of

atropine and other tropane alkaloids. The compound was separated on a stainless-steel column (1 meter X 4.6 mm) packed with sil-X absorbent with 28% aq.  $\text{NH}_3$ -tetrahydrofuran (1:100) as solvent and with a column inlet pressure of 500 lb per sq. in. A different refractive index detector and a UV detector operating at 254 nm were used to monitor the eluate. When applied quantitatively, recoveries of atropine sulphate added to various alkaloid samples were between 88 and 94.5% at the  $\approx 25 \mu\text{g}$  level.

Fell et al. (142) have reported an analysis of atropine sulphate and its degradation products by reversed-phase high-pressure liquid chromatography. Atropine was determined on a column of Hypersil ODS ( $5 \mu\text{m}$ ) with 50 mM. Sodium acetate in 10 mM -tetrabutylammonium sulphate (pH 5.5) - acetonitrile (3:1) as mobile phase and detection at 254 nm. The internal standard was p-toluic acid. Atropine was well separated from its degradation products, tropic acid, atropic acid and apoatropine.

Van Buuren et al. (143) have published a reversed-phase liquid chromatography of basic drugs including atropine - with a fluorogenic ion-pair extraction detector.

Lawrance et al. (144) have separated atropine from other basic organic compounds by continuous post-column ion-pair extraction detection in normal-phase chromatography. The column (6 cm X 3 mm) of LiChrosorb Si 60 ( $5 \mu\text{m}$ ) with a mobile phase ( $1 \text{ ml min}^{-1}$ ) of 10% methanol solution in chloroform containing 0.1 M - butyric acid. Detection of the fluorescence of the organic phase was measured at 452 nm.

#### 9.6.4 Ion-Exchange Chromatography

Morphine sulphate was separated from atropine sulphate by the ion-exchange chromatography technique (145). Determination was done by measuring the ultraviolet absorption at 258 m $\mu$ . E 1% 1 cm = 40. The two drugs cannot be separated

on a weakly basic resin, which converts both to the free alkaloids, but the alkaloids can be separated on a strongly basic resin which retains only the (phenolic) morphine. The procedure of the separation have been described as follows:

Dilute the sample (containing 400 mg of morphine sulphate and 10 mg of atropine sulphate, to 50 ml with 75 per cent methanol. To determine the concentration of morphine sulphate, dilute a 10 ml aliquot to 1000 ml with water and measure the extinction at 285 mμ. To determine the concentration of atropine sulphate, pass a 25 ml aliquot through a two-bed column containing Amberlite IR-4B (10 ml) above Amberlite IRA-410 (10 ml), elute with 75 per cent methanol (4 X 10 ml) and titrate the elute with 0.02 N HCl with bromothymol blue as indicator.

#### 9.6.5 Gas Chromatography

Clarke (21) describes the following three systems for the separation of atropine:-

- a) Column: 1% SE-30 on 100-120 mesh Anakrom ABS. 6 ft X 4 mm internal diameter borosilicate glass column. Column temperature: 180°. Carrier gas: Argon. Gas flow: 65 ml per minute at 180. Detector: Argon ionisation detector or flame ionisation detector. Retention time: 3.22 min. relative to diphenhydramine.
- b) Column: 3% QF-1 on 100-120 mesh Anakran ABS, Column temperature: 200°. Carrier gas: Argon. Gas flow: 80 ml per minute. Other conditions are as in system a. Retention time: 3.80 min. relative to diphenhydramine.
- c) Column: 5% SE-30 on 60-80 mesh Chromosorb W AW. 5 ft X 1/8 inch internal diameter stainless steel column. Column temperature: 230°. Carrier gas: nitrogen. Gas flow: 30.7 ml per minute. Detector: flame ionization detector, hydrogen 22 ml per minutes. Retention time: 0.59 min relative to codeine.

Santoro *et al.* (146) have reported a selective determination of belladonna alkaloids by gas liquid chromatography. Atropine was determined in pharmaceutical preparations in the presence of certain amines. After extraction, the residue is dissolved in dichloromethane and injected glc on a glass column (4 ft X 4 mm) containing 3% OV-17 on Gas-Chrom Q (80 to 100 mesh) operated at 210° with Helium as carrier gas (50 ml per min) and flame ionization detection and measure the peak heights.

Nishimoto *et al.* (147) have described a simplified quantitative analysis of atropine and other alkaloids in scopolia extract. Analysis is carried out by glc on columns (1 mm X 3 mm) packed with 0.75% of Dexsil 300 GC on Gas Chrom Q, with nitrogen (40 ml min<sup>-1</sup>) as carrier gas; with the column at (usually) 180°, atropine (as its trimethylsilyl derivative is separated from hyoscyne, apoatropine and homatropine (the internal standard. With the column at 90° and the carrier gas flow at 30 ml min<sup>-1</sup>, atropine is eluted in about 7 minutes. Thermon 1000 was also used as a stationary phase and diphenhydramine is used as internal standard. The method is applied to gastrointestinal drugs as well as extracts of scopolic roots. The peak height ratio vs atropine content is rectilinear for 25 to 75 ng of atropine.

#### Other GC methods

Atropine tablets were extracted with chloroform in an alkaline media and analyzed using a GC method with diphenhydramine as the internal standard (148).

### 9.6.6 Column Chromatography

Kamienski and Puchalka (149) have reported the separation of atropine and hyoscyamine by a potentiometric chromatographic method. The alcoholic extracts from the leaves Datura stramonium and the roots of Atropa belladonna were diluted until their alkaloid concentration approximately reached 0.001 M. The separation of atropine and hyoscyamine in these solutions



was studied. Four ml of each solution were placed on alumina columns and eluted with either aqueous ethanol (60 or 80%) or a mixture of benzene; water and ethanol (14.5%, 8.5% and 77% respectively), the antimony microelectrode being used to measure potential change in the eluted solution against the volume of the eluate. The most efficient separation was achieved with the benzene - ethanol eluting solution, and a 20 cm column of Merck's alumina.

#### 9.6.7 Paper Electrophoreses

Atropine and hyoscyne were separated quantitatively by paper electrophoreses (150). They were separated with 0.1 N aq.  $\text{NH}_3$  as the electrolyte, and detected as brown spots by exposure to iodine vapour. After elution of the spots, the solvent was evaporated, the residue was nitrated with fuming  $\text{HNO}_3$ , then dissolved in dimethylformamide and tetraethylammonium hydroxide was added according to the method of Freeman (98). The extinction ( $y$ ) of each solution at 545 m $\mu$  was measured and the concentration of each alkaloid is calculated from a given equation.

#### 9.7. Radio-immunoassay

By using  $^3\text{H}$  atropine as tracer, an antiserum was raised by immunisation of rabbits with an immunogen prepared by coupling to human serum albumen. The detection of down to 9 nM atropine in 0.1 ml of serum or plasma is possible. The recovery of atropine added at various concentration to pooled normal human plasma was near 100%. Atropine reacts with the antibodies; other structurally related drugs and atropine-hydrolysis products (tropine and tropic acid) do not interfere. The usefulness of this method in pharmacokinetics studies have been demonstrated in assays of atropine in serial serum samples from two patients who recieved 1.3 mg of atropine in connection with anaesthesia (151).

- A precise, sensitive and rapid radioimmunoassay for the analysis of atropine from unpurified ethanolic extracts of atropine belladonna is described (152).

- Fasth et al. (153) described the first radioimmunoassay for atropine using rabbit antiserum.
- Wurzburger et al. (154) reported a sensitive and specific radioimmunoassay for atropine and showed clearance curve for drug plasma.
- Radioimmunoassay (RIA) was applied to measure atropine in human plasma using antiserum, the plasma clearance of atropine in four adult volunteers was measured.

The measurement accomplished by a competitive RIA using rabbit anti-atropine antibody. Tritiated atropine is used as the radioligand (155).

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# ISOPROTERENOL

Mohammad Tariq and Abdullah A. Al-Badr

*King Saud University*

*Riyadh, Saudi Arabia*

1. Foreword, History, and Therapeutic Category	392
2. Description	392
2.1 Nomenclature	392
2.2 Formulae	393
2.3 Molecular Weight	394
2.4 Elemental Composition	394
2.5 Appearance, Color, Odor, and Taste	394
3. Physical Properties	394
3.1 Melting Point	394
3.2 Solubility	394
3.3 Acidity	395
3.4 Moisture Content and Hygroscopicity	395
3.5 Dissociation Constants (pKa)	395
3.6 Stability	395
3.7 Storage	395
3.8 Sterilisation	395
3.9 Spectral Properties	395
4. Synthesis	404
5. Pharmacokinetics	405
5.1 Absorption, Distribution, Metabolism, and Excretion	405
5.2 Drug Interactions	407
6. Toxicity and Side Effects	407
7. Methods of Analysis	408
7.1 Identification Tests	408
7.2 Chromatographic Methods	409
7.3 Radiographic Method	416
7.4 Spectrophotometric Methods	417
References	420

## 1. Foreword, History and Therapeutic Category

Isoproterenol is the most active of the sympathomimetic amines that act almost exclusively on  $\beta$ -receptors by virtue of their N-alkyl substitution (1). Isoproterenol salts came into use after United States technical investigator studied these compounds which were used in Germany during World War II (2). Isoproterenol is more potent than adrenaline in increasing the rate and force of contraction of the heart (3). The principal clinical utility of isoproterenol is as a bronchodilator, and is effective in relieving bronchial asthma (2).

## 2. Description

### 2.1 Nomenclature

#### 2.1.1 Chemical Names

4-[1-Hydroxy-2-[(1-methylethyl)-amino]ethyl]-1,2-benzenediol;

3,4-dihydroxy- $\alpha$ -[(isopropylamino)methyl] benzyl-alcohol;

$\alpha$ -(isopropylaminomethyl) protocatechuyl alcohol;

1-(3,4-dihydroxyphenyl)-2-isopropylaminoethanol;

Isopropylaminomethyl-(3,4-dihydroxyphenyl) carbinol;

N-isopropyl- $\beta$ -dihydroxyphenyl- $\beta$ -hydroxyethylamine;

dl- $\beta$ -[3,4-Dihydroxyphenyl]- $\alpha$ -isopropylamino-ethanol;

$\beta$ ,3,4-Trihydroxy-N-isopropylphenethylamine;

4-[1-hydroxy-2-[(1-methylethyl) amino] ethyl]-1, 2-benzenediol;

Dihydroxyphenylethanol isopropylamine;

N-isopropylnoradrenaline;

Epinephrine isopropyl homolog;

A21

## CAS Registry Numbers:

- Isoproterenol hydrochloride [51-30-9].
- Isoproterenol sulfate:-
  - anhydrous [299-95-6].
  - dihydrate [6700-39-6]. (4,5,6)

2.1.2 Generic Name

Isoprenaline;  
 Isoproterenol; Isopropylarterenol; Isopropylnor-  
 adrenaline; Isopropylnorepinephrine. (7)

2.1.3 Trade Namesa- Isoprenaline hydrochloride

Proprietary Names: Aerotrol, Aludrine hydro-  
 chloride, Asdrin, Isuprel hydrochloride,  
 Isupren, Isovon, Proternol, Saventrine,  
 Suscardia.

b- Isoprenaline sulphate

Proprietary Names: Aleudrine, Bronchomister,  
 Dyspnoesan, Isomist, Medihaler-ISO, Meterdos-  
 iso, Neo-epinine, Norisodrine, Prenomiser  
 (4,7 & 8).

c- Other Names

Respifral, Billasthman, Isorenin, Asiprenol,  
 Asmalar, Novodrin, Neodrenal, Lomupren,  
 Isopropydrin, Vapo-N-ISO, Isonorin, Assiprenol  
 (4).

2.2 Formulae2.2.1 Empirical

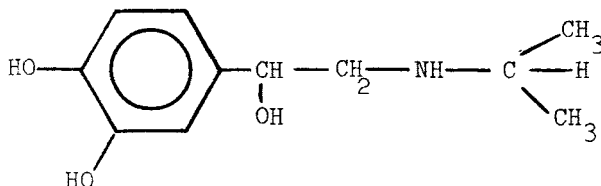
$C_{11}H_{17}NO_3$  (base)  $(C_{11}H_{17}NO_3)_2 \cdot H_2SO_4 \cdot 2H_2O$  Sulfate  
 dihydrate.

$(C_{11}H_{17}NO_3)_2 \cdot H_2SO_4$  anhydrous

$C_{11}H_{17}NO_3 \cdot HCl$  (4)



### 2.2.2 Structural



### 2.2.3 Wiswesser Line Notation

QR BQ DYQIM

Y & GH. (9)

### 2.3 Molecular Weight

211.24 (base) 247.72 (HCl salt) 556.62 (sulfate dihydrate). (4,8 & 9).

### 2.4 Elemental Composition

C, 62.54%; H, 8.11%; N, 6.63%; O, 22.72% (4).

### 2.5 Appearance, Color, Odor and Taste

White to nearly white, odorless, crystalline powder, having a slightly bitter taste; gradually darkens on exposure to air and light; when exposed to air, solutions become pink to brownish pink on standing and almost immediately so when rendered alkaline (5 & 12).

## 3. Physical Properties

### 3.1 Melting Point

168°, 155.5°, 165-170°, 166-170°. (10, 4, 5 & 12, 11).

### 3.2 Solubility

Soluble at 20° in less than 1 part of water and in 55 parts of alcohol, practically insoluble in ether and in chloroform (4).

### 3.3 Acidity

pH of 1% aqueous solution about 5. Aqueous solution turns brownish-pink upon prolonged exposure to air or upon addition of alkali (10).

### 3.4 Moisture Content and Hygroscopicity

Not more than 1%, determined by drying in vacuum (10).

### 3.5 Dissociation Constants (pKa)

8.6, 10.1, 12.0 (20°). (10)

### 3.6 Stability

Isoproterenol gradually darkens on exposure to air and light. Aqueous solutions become pink to brownish pink on exposure to air, and almost immediately when made alkaline. Solutions should contain an antioxidant such as sodium metabisulphite and contact with metals should be avoided as it causes discoloration and loss of activity. (10)

### 3.7 Storage

It should be stored in air tight containers, protected from light. (10)

### 3.8 Sterilisation

Solutions for injection are sterilised by filtration. (10)

### 3.9 Spectral Properties

#### 3.9.1 Ultraviolet Spectrum

In 0.1 N hydrochloric acid maxima at 222 nm (E1%, 1 cm = 257) and 281 nm (E1%, 1 cm = 113); in water, maximum at 280 nm (E1%, 1 cm = 100). (10)

Isoprenaline in 0.1 N sulphuric acid, maxima at 222 mμ (E1%, 1 cm 228), 278.5 mμ (E1%, 1 cm 107), 280 mμ (E1%, 1 cm about 132); minimum at 249.5 mμ. (7)

The ultraviolet spectrum of isoproterenol in methanol solution in the region of 200 to 400 nm

exhibits absorption maxima at 224 and 282 nm. The spectrum is shown in Fig. 1 and was obtained from Sp-100 Pye Unicam UV-Vis Spectrophotometer.

### 3.9.2 Infrared Spectrum

The infrared spectrum of isoproterenol is presented in Fig. 2. The spectrum was obtained as KBr disc, and is recorded in Perkin-Elmer spectrophotometer model 580 B. The frequencies and their structural assignments are as follows:

<u>Frequency (cm<sup>-1</sup>)</u>	<u>Assignments</u>
3000-3400	OH stretch and NH stretch
1610 )	
1620 )	C=C stretch (aromatic)
1540 )	
1460	C-H of CH <sub>2</sub> deformation
1050	C-O of OH groups.

### 3.9.3 Mass Spectrum

The mass spectrum of isoproterenol is shown in Fig. 3, and was recorded on Finnigan -MAT1020 mass Spectrometer. The sample was introduced by direct probe. The spectrum obtained shows a molecules ion peak (M<sup>+</sup>) at m/e 211 and a base peak at m/e 72. The ions, relative intensities and possible structure for these ions are shown below:-

<u>m/e</u>	<u>Relative intensity %</u>	<u>Fragment</u>
211	2	M <sup>+</sup> .
72	100	CH <sub>2</sub> = <sup>+</sup> NH-CH(CH <sub>3</sub> ) <sub>2</sub>
43	14	CH(CH <sub>3</sub> ) <sub>2</sub> <sup>+</sup>

### 3.9.4 Nuclear Magnetic Resonance Spectra

#### 3.9.4.1 Proton Spectrum

The proton NMR spectrum of isoproterenol

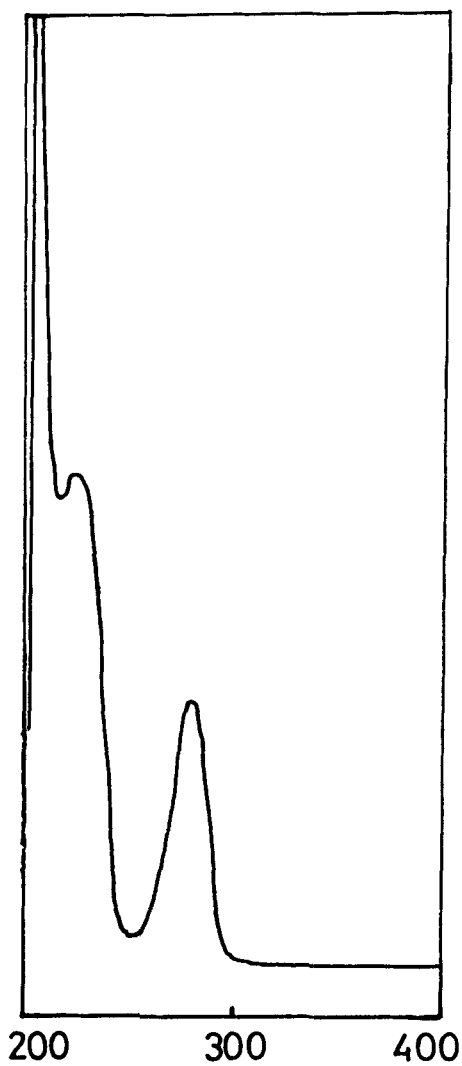


Fig. 1 Ultraviolet Spectrum of Isoproterenol in Methanol.

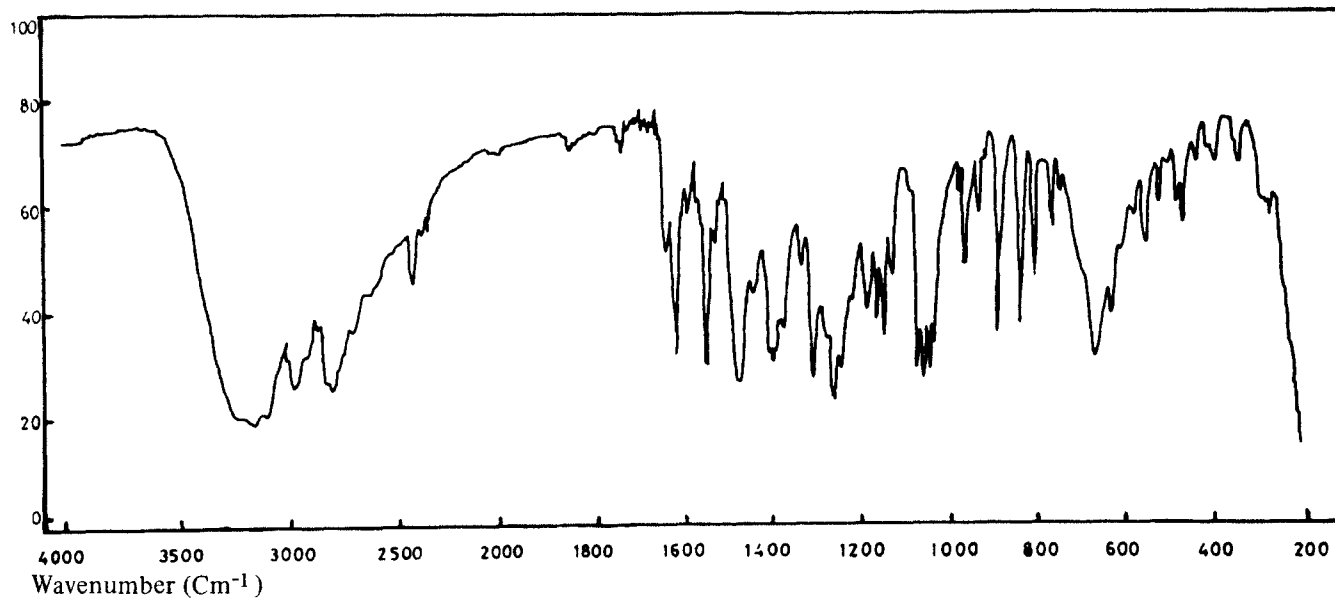


Fig. 2: Infrared Spectrum of Isoproterenol (KBr disc).

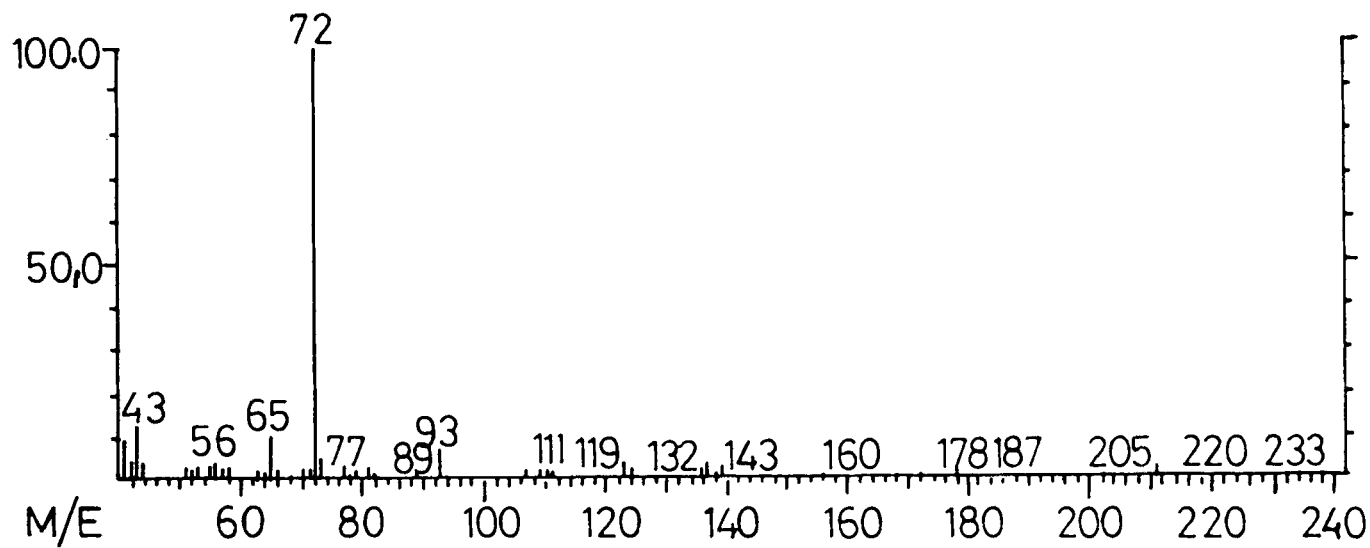


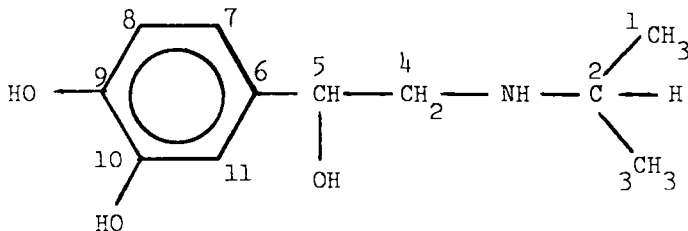
Fig. 3. Mass Spectrum of Isoproterenol

was recorded on Joel-XL-100-NMR spectrometer with  $D_2O$  as a solvent and DSS (2,2-dimethyl-2-silapentane-5-sulphonate) as internal reference. The spectrum is shown in Fig. 4. The signals are assigned as follows:

<u>Proton</u>	<u>Chemical Shift</u>	<u>Multiplicity</u>
Phenyl protons	6.8 - 7	multiplet
- <u>CH</u> -OH	4.9	triplet
- <u>CH<sub>2</sub></u> -	3.25	doublet
- <u>CH</u> (CH <sub>3</sub> ) <sub>2</sub>	3.4	multiplet
-CH <sub>3</sub>	1.34	doublet
-OH	exchanged	-
-NH	exchanged	-

#### 3.9.4.2 Carbon-13 Spectrum

The noise-decoupled and off-resonance carbon-13 NMR spectra of isoproterenol are shown in Fig. 5 and 6 respectively. The spectra were obtained on a Joel-XL-100 NMR spectrometer. The spectral assignments are listed below:



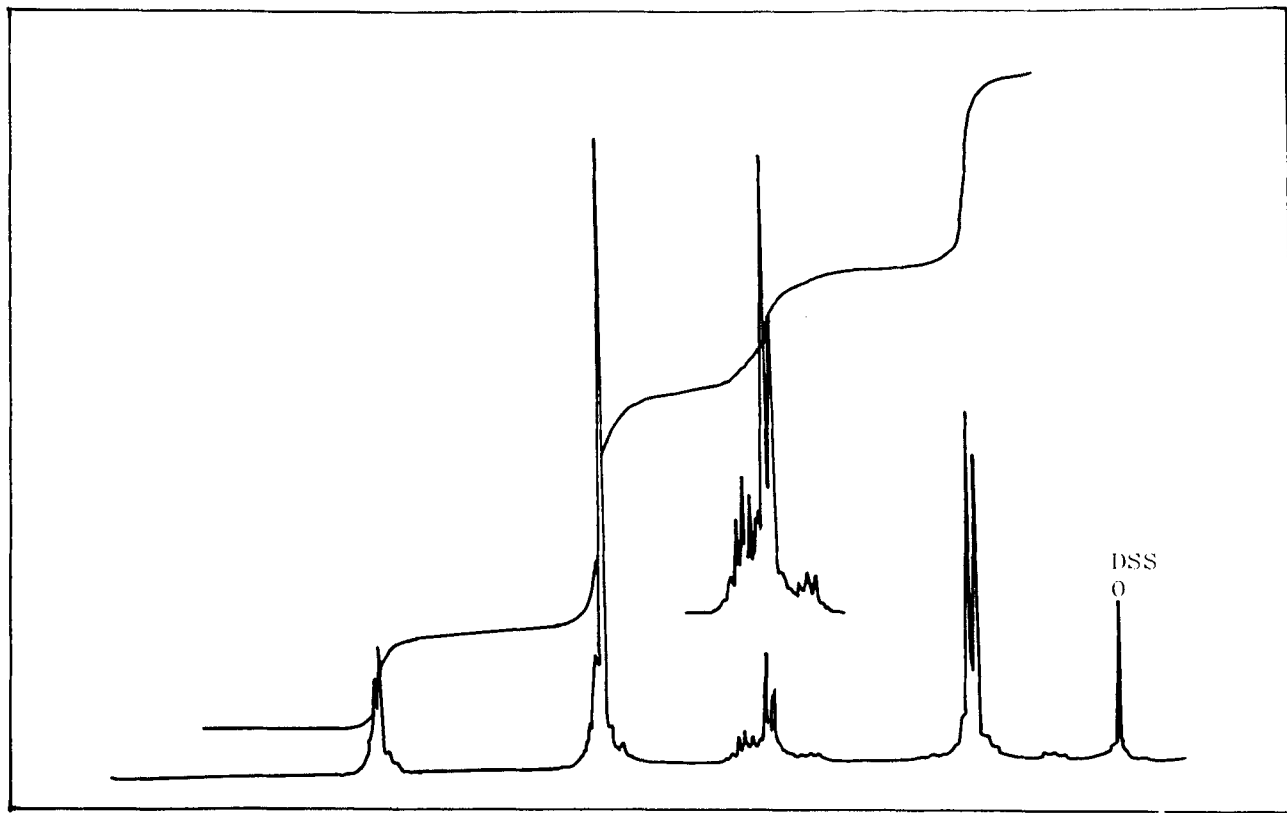


Fig. 4: Proton NMR Spectrum of Isoproterenol in D<sub>2</sub>O.



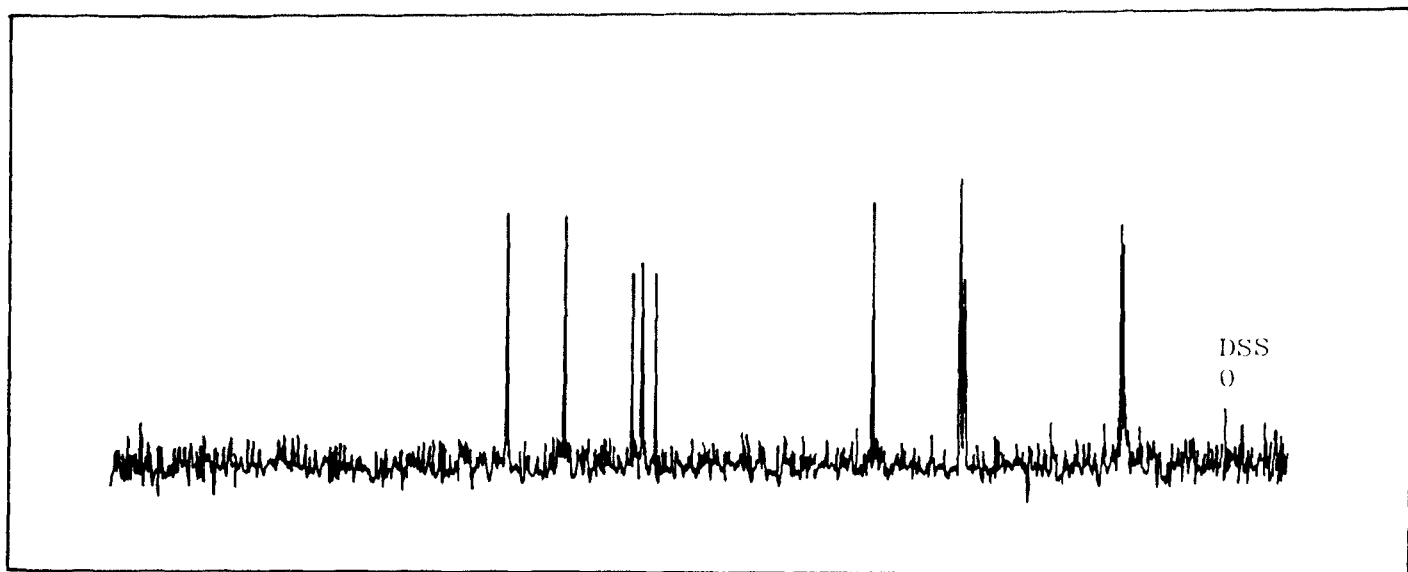


Fig. 5: Carbon-13 NMR Spectrum of Isoproterenol in D<sub>2</sub>O (noise-decoupled).

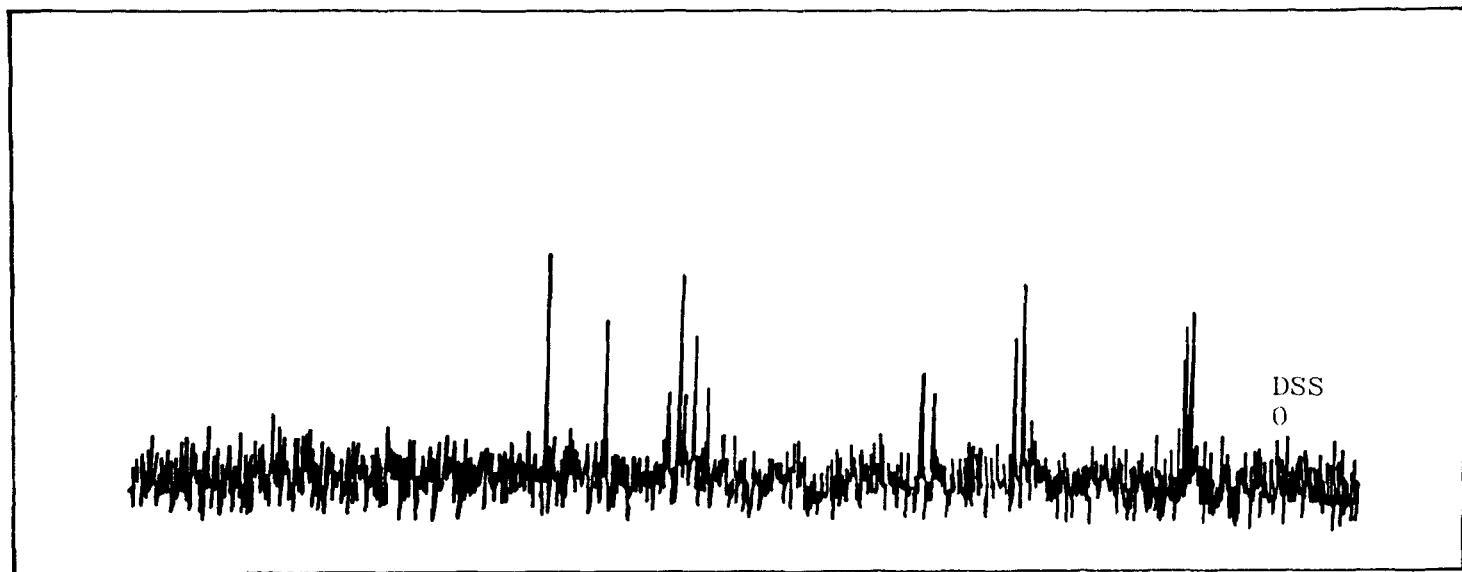


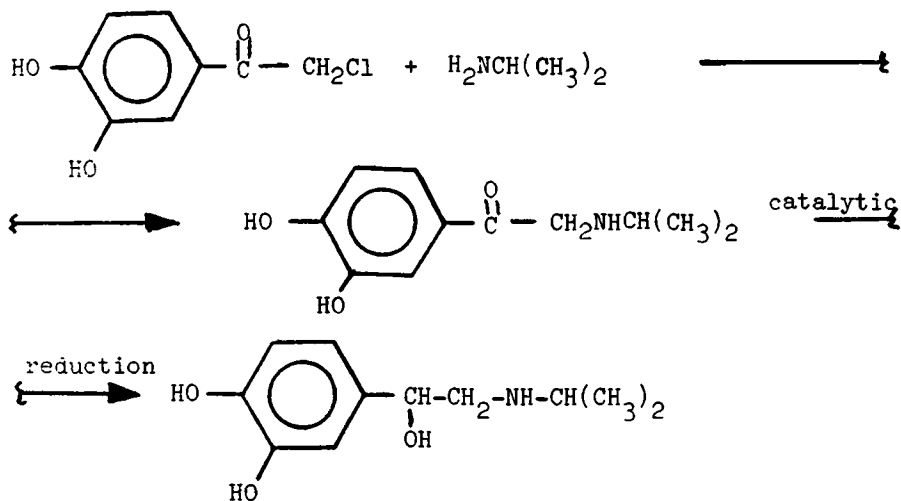
Fig. 6: Carbon-13 NMR Spectrum of Isoproterenol in  $D_2O$  (off resonance).

<u>Carbon No.</u>	<u>Chemical shift (ppm) relative to DSS</u>	<u>Multiplicity</u>
1 and 3	20.96	doublet
2	53.66	multiplet
4	52.84	doublet
5	71.45	triplet
6	134.97	singlet
7	121.17	doublet
8	118.97	doublet
9 and 10	146.83	singlet
11	114.42	doublet

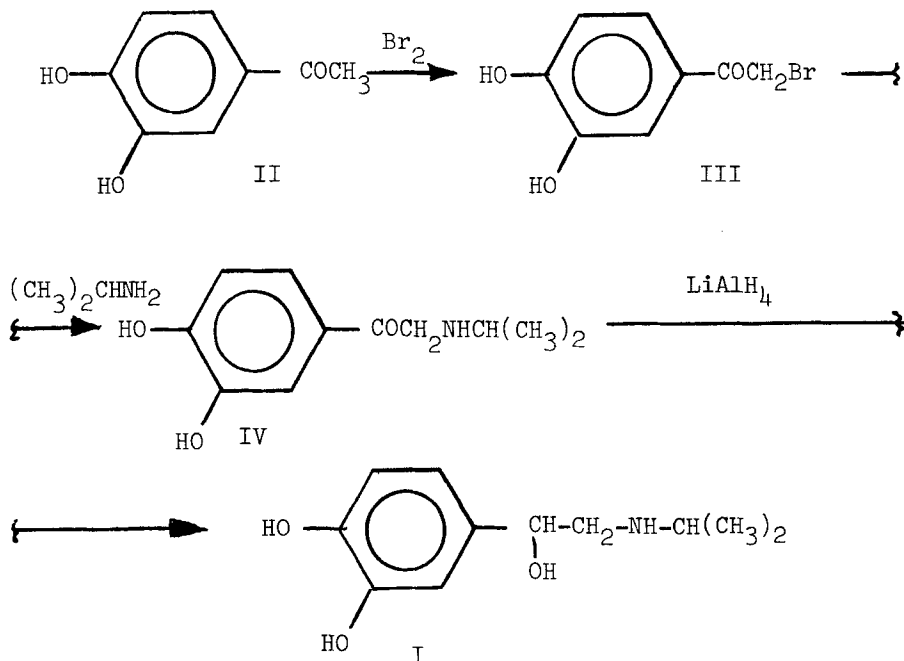
#### 4. Synthesis

a) Isoproterenol can be prepared by condensing 3,4-dihydroxy- $\alpha$ -chloroacetophenone and isopropylamine to form 3,4-dihydroxyisopropylaminoacetophenone. This, on catalytic reduction gives the required product. The detailed sequence of the reactions is given and has been described here schematically in brief (13).

Scheme I



- b) Isoproterenol and its analogues can be prepared by the method reported by Kaiser and coworkers, (14). 1,2-dihydroxyacetophenone (II) was brominated with bromine in presence of 2-pyrrolidinone to give 2-bromoacetophenone (III). This, on condensation with isopropylamine yielded the aminoketone (IV), the reduction of compound (IV) with lithium aluminum hydride afforded the required isoproterenol (I).



## 5. Pharmacokinetics

### 5.1 Absorption, Distribution, Metabolism and Excretion

Isoproterenol is readily absorbed when given parentally or as an aerosol. Absorption of sublingual or oral doses is unreliable (1). The drug is poorly absorbed from stomach, but well absorbed from small intestine, proximal colon, rectum and from the mucous membrane of the trachea (15). It is recognized that the dosage requirements for isoproterenol vary widely according to the route of administration; when the drug

is given intravenously pharmacological effects are seen with only few micrograms (16 & 17), whereas using the oral route, tablets containing 180-360 mg are required daily to control chronic heart block (18). Teixeira et al. (19) reported 14-18% plasma protein binding of isoproterenol, suggesting a very low affinity and capacity of isoproterenol to bound with plasma proteins. Conolly et al. (20) showed that most of the drug administered orally was metabolised during absorption and that this metabolism probably occurred in the wall of intestines. Pharmacokinetic analyses have shown that isoproterenol undergoes extensive metabolic degradation at first by-pass after oral administration. the onset of action after oral administration of isoproterenol occurs after 20 minutes and the effect lasts for about 60 minutes. Absorption is quicker after intramuscular injection and by inhalation. After a dose of 5 mg, administered as an aerosol inhalation, a plasma concentration of about 0.03 ngm/ml is attained in 5 minutes. Conway et al. (15) reported that isoproterenol disappears from the circulation within few minutes. The plasma half-life after rapid intravenous injection is 3-5 minutes and often intravenous infusion is given for about 2 hours. More detailed studies in children show the decline in plasma concentration to be biphasic, the  $\alpha$ -phase having half-life of 2-5 minutes and the  $\beta$ -phase a half-life of 3-7 hours (10).

After oral administration or inhalation most of the drug is conjugated by O-methylation and sulphate conjugation but after intravenous or intra-bronchial administration, it is mostly metabolized to O-methylated form (21). A major amount of orally administered isoproterenol is metabolized by conjugation by the gut wall prior to absorption. The extent of conjugation may be reduced by treatment with salicylamide, the sulphate conjugate is inactive. The methylated metabolite by catechol-O-methyl-transferase to a 3-methoxy derivative has a weak antagonistic activity on  $\beta$ -receptors. Rats and guinea pigs inactivate isoproterenol within minutes of intravenous injection and the metabolites are excreted in urine and bile (22). In the dogs isoproterenol is metabolized very rapidly and less than 1% of the dose is eliminated unchanged in the urine (16). The excretion of isoproterenol greatly depends upon the route of administration. About 90% of the intravenously administered isoproterenol is excreted in urine in 24 hours; mostly as the 3-O-

methyated metabolites, with about 15% being excreted unchanged (23). Following inhalation or oral administration of isoproterenol, 80-95% of dose is excreted in urine, mainly as sulphate conjugate, 1-2% as unchanged drug and 1-2% as free methyated metabolite, small amounts of the dose are excreted in the bile, mainly as methyated metabolite. In children, after intravenous administration, 75% of unchanged drug is present in a 5-minute urine sample (23).

## 5.2 Drug Interactions

### 5.2.1 Drug-Drug Combinations

The following drugs may change (or interact) the pharmacologic effects of isoproterenol, when used concomitantly.

- a) Amitriptyline    b) Nortriptyline    c) Propranolol
- d) Monoamine oxidase inhibitors (24, 25 & 26).

### 5.2.2 Compatibilities

Isoproterenol is compatible with all available intravenous fluids except sodium bicarbonate, and when mixed with the following drugs. (27)

- a) Heparin sodium                      b) Tetracycline HCl.
- c) Vitamin B complex injection with vitamin C.
- d) Calcium chloride                    e) Cephalothin sodium injection
- f) Multi-vitamin                        g) Magnesium sulfate infusion.
- h) Potassium chloride                i) Succinylcholine chloride
- j) Oxytetracycline HCl.

### 5.2.3 Incompatibilities

Isoproterenol is incompatible when mixed with the

- a) Sodium bicarbonate    b) Aminophylline
- 5% solution. (28)

## 6. Toxicity and Side Effects

Palpitation, tachycardia, headache, and flushing of the skin are common; anginal pain, nausea, tremor, dizziness, weakness, and sweating are less frequent. Cardiac arrhyth-

mias can occur readily, although they are not usually serious. Cardiac arrest can occur when the heart is subjected to an increased work load (1).

Van Metre (29) described severe refractory wheezing in patients receiving large amounts of isoproterenol aerosol (5-60 times normal dose). Ball (30) reported a case of discoloration and destruction of permanent incisor and canine teeth in a 12 year old girl who received oral isoproterenol over a period of 6 years. Depot preparations given sublingually can cause buccal ulceration (31).

Excessive use of isoproterenol inhalants containing a fluorocarbon-propellant has led to sudden death by unknown mechanisms (5). For children the minimum lethal dose is 100 mg, when applied to mucous membranes. LD<sub>50</sub> in mice is more than 2 g/kg orally (7).

## 7. Methods of Analysis

### 7.1 Identification Tests

Several methods have been used for the qualitative analysis of isoproterenol:

- i- To 2.0 ml of 1 percent w/v solution of isoproterenol, 2 drops of ferric chloride test-solution is added. An emerald green colour develops which changes upon gradual addition of sodium hydrogen carbonate solution, first to blue and then to red (8).
- ii- To 5.0 ml of 1 percent w/v solution of isoproterenol, 2 drops of silver nitrate solution is added. Shining greyish precipitate is produced within ten minutes and the solution turns pink (8).
- iii- To 1.0 ml aqueous solution containing 10 mg isoproterenol, a drop of phosphotungstic acid solution is added. A white precipitate is formed which turns brown on standing; the presence of isoproterenol (10).
- iv- 1.0 ml of a 1 mg/ml solution of isoproterenol in water is diluted with 10.0 ml of water, 2 drops of 0.1 N HCl and 1.0 ml of 0.1 N iodine is added to it. The solution is allowed to stand for five minutes then 2.0 ml of 0.1 N sodium thiosulfate is

added. Appearance of red-brown colour indicate the presence of isoproterenol (10).

## 7.2 Chromatographic Methods

### 7.2.1 Paper Chromatography

Clarke (7) has described a method of identification by ascending chromatography on Whatman No. 1 paper, buffered by dipping in a 5% solution of sodium hydrogen citrate and dried at 25°C for 1 hour. The solvent system consisted of 4.8 gm citric acid dissolved in a mixture of 130 ml of water and 870 ml of n-butanol. The R<sub>f</sub> is found to be 0.22. Blue fluorescence is observed under UV light at 254 mμ. Bromocresol green spray is used as the location reagent.

### 7.2.2 Thin Layer Chromatography

- i- Clarke (7) has also described a TLC method for isoproterenol identification on glass plates coated with silica gel G, 0.25 mm thick, using strong ammonia solution: methanol (1.5 : 100). The R<sub>f</sub> is found to be 0.33 when sprayed with potassium permanganate solution.
- ii- Halmekoski and Kemistilehti (32) have studied and reported the behaviour of isoproterenol in different, stationary and mobile phases, (Table (1)). Using Kieselgel G buffered to pH 4. Kieselgel G treated with Na<sub>2</sub>MoO<sub>4</sub>, Kieselgel G treated with Na<sub>2</sub>WO<sub>4</sub> and Kieselgel treated with borax. The following table illustrates the movement of isoproterenol in different environments. Folin - Denis reagent is used to locate the isoproterenol.

### 7.2.3 Thin-Layer Radiochromatography

Isoproterenol and its metabolites in biological fluids have been separated and identified by thin-layer radiochromatography. Using dl-isoproterenol -7-<sup>3</sup>H HCl, Kadar *et al* (23) reported such a method in which the radioactivity was counted in a scintillation counter. Precoated cellulose plates were used and were run in two different



TABLE I

LAYER SOLVENT	L <sub>1</sub>	L <sub>2</sub>	L <sub>3</sub>	L <sub>4</sub>	L <sub>1</sub>	L <sub>2</sub>	L <sub>3</sub>	L <sub>4</sub>	L <sub>1</sub>	L <sub>2</sub>	L <sub>3</sub>	L <sub>4</sub>	L <sub>1</sub>	L <sub>2</sub>	L <sub>3</sub>	L <sub>4</sub>
	S <sub>1</sub>	S <sub>1</sub>	S <sub>1</sub>	S <sub>1</sub>	S <sub>2</sub>	S <sub>2</sub>	S <sub>2</sub>	S <sub>2</sub>	S <sub>3</sub>	S <sub>3</sub>	S <sub>3</sub>	S <sub>3</sub>	S <sub>4</sub>	S <sub>4</sub>	S <sub>4</sub>	S <sub>4</sub>
ISOPROTERENOL Rf x 100	45	11	14	20	48	21	34	40	31	17	20	20	53	24	31	46

L<sub>1</sub> = Kieselgel G buffered to pH 4.0.

S<sub>1</sub> = n-butanol saturated with aqueous sulfur dioxide solution (H<sub>2</sub>SO<sub>3</sub>).

L<sub>2</sub> = Kieselgel G treated with Na<sub>2</sub>MoO<sub>4</sub>.

S<sub>2</sub> = n-butanol-acetic acid-H<sub>2</sub>SO<sub>3</sub> (4:1:5).

L<sub>3</sub> = Kieselgel G treated with Na<sub>2</sub>WO<sub>4</sub>

S<sub>3</sub> = n-amyl alcohol-acetic acid - ethanol-H<sub>2</sub>SO<sub>3</sub> (4:1:1:5).

L<sub>4</sub> = Kieselgel G treated with Borax

S<sub>4</sub> = n-butanol-n-propanol-acetic acid-H<sub>2</sub>SO<sub>3</sub> (4:1:1:5).

solvent systems (i) butanol: acetic acid: water (4:1:1) and (ii) secondary butanol: pH 3.9 buffer (4:1). The buffer consisted of water : Pyridine: acetic acid (100:10:41). Non radioactive isoproterenol was added as a carrier. Potassium ferri-cyanide, 0.44%, in 0.1 M phosphate buffer was used as the spraying agent for location. 0.5 cm sections were cut from the plates and eluted with 5 ml methanol and counted in 15 ml. butyl PPD-toluene scintillator fluid (23).

#### 7.2.4 Gas Liquid Chromatography

Watson and Lawrence (33) developed a simple and specific GLC method for the quantitation of isoproterenol in commercial tablets, powders, inhalation solutions, ophthalmic and nasal drops and injectable preparation. Samples are taken to dryness (where required). The dry residue of isoproterenol sample containing 2-18 mg are accurately weighed into five separate 5 ml septum sealed vials, then 2 ml of internal standard solution containing dibenzyl succinate (2.5 mg/ml) in spectro grade dimethyl formamide solution is dispensed in each vial. The samples are now treated with 1 ml of trimethyl silylimidazole and allowed to stand for 30 minutes in the dark at ambient temperature with occasional shaking, 2 micro litre of silylated solution is injected in gas chromatograph (having flame ionized detector) fitted with a 5% O.V-101 on chromosorb 750 (100-120 mesh) U-shaped glass column [1.82 m X 6 mm O.d] preconditioned at 265° for 18 hours. The support is coated using a fluidizer maintained at 150° for 2 hours with a suitable nitrogen flow to ensure gentle, yet thorough, drying of the packing material, the temperature conditions being: column 170° (10 min) and then programmed to 245° at 2°/min; injection port, 225°; and detector, 225°. Gas flow were: nitrogen, 70 ml/min; hydrogen, 40 ml/min; and air, 380 ml/min. The detector signal is fed to an electronic integration with input signal range capacity of 0-Iv. Quantitation of the flame ionization detector signal is achieved related to dibenzyl succinate internal standard on the electronic integrator. The percent recovery of isoproterenol by this method vary between 94.9 - 103.4

percent. Commonly incorporated buffering and antioxidant exceptient compounds did not interfere in this analysis.

## 7.2.5 High-Pressure Liquid Chromatography

### 7.2.5.1 Ion-Pair HPLC Analysis

A large number of HPLC methods have been developed for the quantitative determination of isoproterenol.

a- Ghanekar and Gupta (34) have devised an ion-paired high pressure liquid chromatography method to determine isoproterenol in partly degraded solution. Chromatographic conditions: The high pressure liquid chromatograph capable of operating of inlet pressure of upto 6000 psig. is used. The multiple wavelength detector was set at 280 nm for maximum absorption, the detector is attached to a recorder and an integrator. A column of 30 cm long and 4 mm i.d., is used. A 20% v/v solution of methanol in water containing 2% acetic acid may be used with or without 0.005 M sodium 1-heptane sulfonate, the pH of both solvents is adjusted to  $2.6 \pm 0.05$ . The flow rate must be 1.6 mm/min (inlet pressure approximately 1500 psig). The absorption unit for full scale deflection is 0.04. The chart speed is fixed at 30.5 cm/hour. Procedure: a 0.1% solution of isoproterenol is prepared. For decomposition, 5 ml of isoproterenol stock solution is mixed with 5 ml of sodium hydroxide solution (0.01N). After an appropriate period the reaction mixture is quenched by adding 5 ml sulfuric acid of appropriate concentration. The final volume is made up with distilled water. 10  $\mu$ l of this solution is injected to the column using above mentioned solvents and conditions for comparison 10  $\mu$ l of standard solution containing 0.5 to

1.5  $\mu$ l of isoproterenol. The results are calculated by directly comparing the peak areas using the formula:

$$\text{Percent of claim} = \frac{A_a}{A_s} \times 100$$

where  $A_a$  is peak area of test sample and  $A_s$  is peak area of standard.

- b- Clements et al (35) described an ion-paired HPLC method to determine isoproterenol in the presence of an impurity, degradation product and anti-oxidant. The apparatus included a modular chromatograph consisting of a twin head reciprocating pump with pulse-dampering and a column (100 X 4.5 mm i.d.) packed with ODS-Hypersil-5  $\mu$ m. The variable wavelength UV detector is set at 280 nm, the detector signals are recorded on a potentiometric recorder and monitored by integrator. Isoproterenol hydrochloride solution (0.1%) is freshly prepared in water immediately before use. A solution of N-Isopropyl noradrenolutin is prepared by the addition of 5 ml of 10% aqueous NaOH solution, 200 ml of the solution of N-Isopropyl-noradrenochrome. The solutions are stored at - 7°C till required.

The 2 ml of isoproterenol hydrochloride solution (0.1%) is mixed with 2 ml of adrenaline acid tartarate solution (0.1%) as the internal standard. An aliquot (2-20  $\mu$ l) is injected into chromatograph and eluted with water-ethanol-methanol-acetic acid - sodium lauryl sulfate (70:30:2:0.002) at a flow rate of 1.4 ml/min (pressure 1100 psig). Standard solution of isoproterenol (0.1-0.8 mg/ml) is chromatographed after addition of internal standard. The retention time of isoproterenol is 9.5 min. Thus, HPLC method is not effected by any of the following substance: degradation (N-

isopropylnoradrenochrome, N-isopropylnoradrenolutin); impurities (isoproterenone); the sulphonic acid derivative formed with sodium metabisulphite; antioxidants (ascorbic acid, sodium bisulphite, disodium EDTA) in fresh or autoclaved solutions.

#### 7.2.5.2 Reversed-Phased HPLC

Causon et al (36) described a rapid, sensitive and precise method for the determination of isoproterenol in plasma and urine suited to pharmacokinetic studies in man. The liquid chromatograph; an Altex 100A solvent delivery pump and Altex 210 injection valve fitted with 100  $\mu$ l sample loop and a 150 X 4.6 mm i.d. stainless steel column packed with 5  $\mu$ m diameter Altex ultrasphere octyle particles. The detection system comprised the amperometry detector fitted with a glassy carbon electrode assembly. Radiochromatogram is obtained using suitable radio scanners.

The mobile phase consisted of citrate-phosphate buffer adjusted to pH 6, containing 3% v/v methanol and disodium salt of EDTA at final concentration of 2 mM. The flow rate continuously maintained at 0.1 ml/min however, is increased to 1 ml/min prior to sample workup and injection, the amperometric detector is used at sensitivity at 10 nA and a potential difference of + 0.50 V vs. Ag/AgCl reference electrode. For estimation in blood and urine, an oral dose of d-isoproterenol bitartrate (1 mg/kg body weight) given: 24 hours urine sample and blood sample is collected for six hours at 30 minutes interval. Plasma sample separated by centrifugation. 2 ml of plasma or urine is added to 2 ml of 1M HCl and the mixture is placed in boiling water bath for 30 minutes. 2 ml of this hydrolysate were transferred in conical polystyrene

tubes containing 60 mg of activated alumina, 1 ml of 0.1 mM Na<sub>2</sub> EDTA with 1 mM HCl and 100 µl of µg/ml N-methyldopamine (internal standard). The pH is adjusted at 8.6 by adding 1 ml of 3 M Tris-HCl buffer, and the tubes are mixed for 15 minutes. Supernatants are discarded and alumina washed thrice with distilled water. Isoproterenol is eluted in 200 µl of 0.1 M of orthophosphoric acid by mixing for 2 minutes. The supernatant is stored at - 20°C for upto one week or directly injected into HPLC system (50 µl). Measurement of d-isoproterenol is done by comparison with peak height ratio of isoproterenol to N-methyldopamine in the sample.

#### 7.2.5.3 HPLC - Trihydroxyindole Method

Kishimoto et al (37) described a simple method for determination of isoproterenol by HPLC coupled with an automated trihydroxyindole method. The sensitivity is 0.2 p mol and recovery in plasma and urine is 89% and 101%. To the deproteinized plasma, 20 µl of working standard was added to make the final concentration of isoproterenol in the range of 1-240 p mol/ml. In case of urine 100 µl of HCl and 10 µl of working standard solution is added to make the final concentration of isoproterenol in the range of 20 p mol/ml - 4.8 nmol/ml. For HPLC analysis 200 µl deproteinized plasma/or 10 µl of urine is injected in the column with a flow rate of 1.0 ml/min and the temperature at 40°C (column size 150 cm X 2.1 mm i.d. filled with cation exchange Zipex - SCX, particle size 30 µm). Isoproterenol in the column is converted into trihydroxyindole derivative automatically and the fluorescence intensity is measured with a spectrofluorometer with the excitation and emission respectively. The retention time of isoproterenol is 24 minutes and peak is well separated from interfering substances in urine and deproteinized plasma.

#### 7.2.5.4 HPLC Determination in Intravenous Solutions

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Williams et al (38) established an ion-pair HPLC method, that is rapid and specific for the determination of isoproterenol in intravenous solutions with aminophylline, using spectrofluorometer detector. Standard solution of isoproterenol hydrochloride (0.2 - 2.4 µg/ml) is freshly prepared by diluting stock solution with 5% dextrose in water for injection. The mobile phase being 0.35 M acetic acid and 0.005 M sodium heptane sulfonate in methanol. The flow rate is adjusted between 1.6 to 2.0 ml/min at ambient temperature. Detection is made using excitation wavelength of 285 nm and emission 315 nm. Good reproducibility is obtained without using an internal standard. This method has been successfully applied as a stability indicating method for determining the rate and percentage of autooxidation of commercial preparations.

#### 7.3 Radiographic Method

Herting, (22) reported a radiographic method of isoproterenol assay for the purpose of pharmacokinetic studies. D1-7-<sup>3</sup>H-isoproterenol is prepared by reduction of isopropyl-noradrenaline with tritium gas, the specific radioactivity being 2.3 c/mmol. 46 micro c of labelled isoproterenol was injected in male rats by tail vein injection. 24 hours urine sample is collected and counted in a Packard liquid scintillation spectrophotometer. The metabolites are separated by paper chromatography using different solvent systems, n-butanol: acetic acid: water (4:1:1), isopropanol: ammonia: water (8:1:1) and n-butanol: ethanol: water (4:1:1). After development of the paper chromatograms, the distribution of radioactivity on paper is determined using chromatogram scanner. The peaks obtained correspond to isoproterenol and its metabolites. Distribution of isoproterenol in different tissues may be measured by sacrificing the animal and extracting different organs with acetate buffer, purifying on alumina column and eluting with 0.4 N acetic acid.

## 7.4 Spectrophotometric Methods

### 7.4.1 Ultraviolet

- a) In a recent study Korany and Wahbi (39) described a method for the estimation of isoproterenol in pharmaceutical products as chloranil is known to form a colored complexes with primary and secondary amino groups of arylamines. In this method, 20 tablets are powdered and extracted with 0.1 M hydrochloric acid, the extract is filtered and diluted to 100 ml with 0.1 M hydrochloric acid. 20 ml of this solution is taken, pH adjusted to 7 with 0.05 M disodium tetraborate and diluted again to 100 ml with water. 2 ml of this neutral solution is taken and 5 ml of chloranil solution, saturated with ethanol and 2 ml of 0.05 M disodium tetraborate solution is added and diluted to 25 ml with water. A blank solution, using 2 ml of 0.05 M disodium tetraborate and 5 ml of chloranil solution, is prepared and diluted to 25 ml with water. The solutions are heated at 65°C for 30 minutes and absorbance is measured at 354 nm.
- b) Based on the reaction of sodium cobaltinitrite with phenolic compounds, Wahbi et al (40) developed a method for the identification and estimation of isoproterenol in pharmaceutical preparations. The method involves the treatment of a 2 ml portion of 25 mg/10 ml solution of isoproterenol in glacial acetic acid with 2 ml glacial acetic acid and 2 ml of sodium cobaltinitrite solution (5%, in water). A blank is prepared with the solvent. These are heated in a boiling water bath for 15 minutes, cooled and diluted to 50 ml with water. The absorbance of yellow colored solution is measured at 425 nm and results are obtained by comparing with the standard curve similarly prepared from a standard curve similarly prepared from a standard isoproterenol solution. These authors found isoproterenol to show a maxima at 325 nm, if the above cobaltinitrate treated solution of isoproterenol, is first diluted with 10 ml water and 20 ml of 20% NaOH, filtered and washed with three 5 ml portions



of water and diluted to 50 ml water.

#### 7.4.2 Colorimetry

- a) Doty (41) developed a colorimetric method for the determination of isoproterenol in pharmaceutical products. The color developed reaches maximum intensity quickly and is constant for some hours. The method is based on the complex forming capability of isoproterenol with iron citrate. To a 10 ml solution of isoproterenol, 0.1 ml of the iron-citrate reagent is added. This is followed by the addition of 1.0 ml of buffer reagent of pH 8.0. After mixing, the solution is allowed to stand for twenty minutes and the color intensity is measured at 540 mu. The concentration of isoproterenol is determined from the standard curve of isoproterenol prepared similarly.
- b) Wahbi et al (42) recently developed a very precise (precision, C.V. = 0.5 %) and accurate colorimetric method to determine isoproterenol with p-benzoquinone at pH 5.4. In their method, to 5.0 ml of a 2 mg/10 ml solution of isoproterenol in buffer solution of 5.4, is added 2 ml of the p-benzoquinone reagent (1% w/v in ethanol). The mixture is allowed to react and stand at room temperature for 30 minutes. Absorbance is then measured at 502 nm against a blank which is simultaneously run using 5 ml of the buffer solution of pH 5.4.
- c) Barker et al (43) have proposed the potassium sulfate reaction as an specific test for the colorimetric determination of isoproterenol and the value of this method has been confirmed by Rees (44). The following is the modified version for the spectrophotometric determination of isoproterenol:

To 1 ml solution, containing 0.01 to 0.10 mg isoproterenol is added the coloring reagent containing 0.2 percent potassium persulfate in water, 1.0 percent sodium chloride, 0.239 percent disodium hydrogen phosphate and 0.937 percent sodium dihydrogen phosphate, main-

tained at pH 5.5. The intensity of the color is immediately measured at 490 mμ in a 1 cm all glass cell. This cell is then placed in a thermostat at 22°C for 30 minutes and the color intensity is read again. The increase in intensity is compared with the standard curve prepared from the standard isoproterenol solution.

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# WARFARIN

Salim A. Babhair, Mohammad Tariq, and  
Abdullah A. Al-Badr

*King Saud University  
Riyadh, Saudi Arabia*

1. History	424
2. Description	424
2.1 Nomenclature	424
2.2 Formulae	425
2.3 Molecular Weight	425
2.4 Elemental Composition	425
2.5 Appearance, Color, Odor, and Taste	425
3. Physical Properties	425
3.1 Melting Point	425
3.2 Extraction	426
3.3 Solubility	426
3.4 Alkalinity	426
3.5 Moisture Content and Hygroscopicity	426
3.6 Dissociation Constant	426
3.7 Storage	426
3.8 Spectral Properties	426
4. Synthesis	437
5. Pharmacokinetics	440
5.1 Absorption, Distribution, Metabolism and Excretion	440
6. Therapeutic and Other Uses	440
7. Toxicity	441
8. Methods of Analysis	442
8.1 Identification	442
8.2 Spectrophotometric Methods	443
8.3 Chromatographic Methods	445
References	450

## 1. History

Ingestion of spoiled sweet clover silage caused hoemorrhagic disorders in cattle (1). The toxic principle which significantly reduced plasma prothrombin and caused hoemorrhage was later identified as bishydroxycoumarin. Subsequently a number of chemical derivatives of the coumarin were synthesized and tested. Ikawa and co-workers (2) synthesized a very potent agent called warfarin which was initially used as a popular rodenticide till 1951. The clinical trials on warfarin were conducted in 1954 and it was recommended as an oral anticoagulent for human use (3).

## 2. Description

### 2.1 Nomenclature

#### 2.1.1 Chemical Names

- \* 4-Hydroxy-3-(3-oxo-1-phenylbutyl)-coumarin.
- \* 3-( $\alpha$ -acetonylbenzyl)-4-hydroxycoumarin.
- \* 3- $\alpha$ -Phenyl- $\beta$ -acetylethyl-4-hydroxycoumarin.
- \* 2H.1.Benzopyran-2-one, 4-hydroxy-3-(3-oxo-1-phenylbutyl).
- \* 1-4'-Hydroxy-3'-coumarinyl)-1-phenyl-3-butanone.
- \* 3- $\alpha$ -Phenyl- $\beta$ -acetylethyl-4-hydroxycoumarin. (4,5 & 6).

#### 2.1.2 Generic Name

Warfarin. WARF Compound 42, Compound 42.

#### 2.1.3 Trade Names

Warfarin sodium:-

- Coumadin.
- Panwarfin.
- Prothromadin; Tintorane;
- Marevan, (Dethomre as Rodenticide);
- Waran, Warfilone, Warnerin.

Warfarin potassium:-

Anthrombine.k. (5 & 8).

#### 2.1.4 Cas Registry No.

(81-81-2) (7).

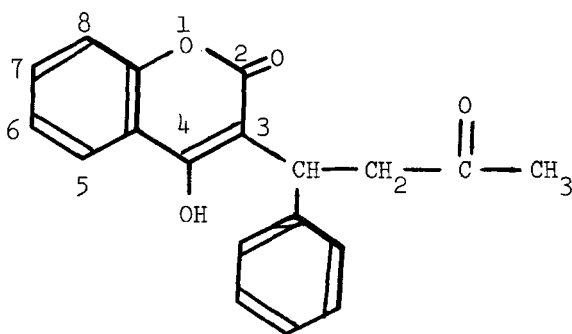
## 2.2 Formulae

### 2.2.1 Empirical

$C_{19}H_{16}O_4$  (Warfarin)

$C_{19}H_{15}NaO_4$  (Sodium salt)

### 2.2.2 Structural



## 2.3 Molecular Weight

308.32

## 2.4 Elemental Composition

C 74.01%, H 5.23%, O 20.76%.

## 2.5 Appearance, Color, Odor and Taste

An odorless white crystalline powder (potassium salt) with a bitter taste. (4)

## 2.6 Crystal Properties

Fatty substance consisting of long well formed needles (8).

## 3. Physical Properties

### 3.1 Melting Point

Melts between  $159^{\circ}$  and  $160^{\circ}$  (5), crystals from alcohol m.p.  $161^{\circ}$ .



### 3.2 Extraction

Warfarin is extracted by organic solvents from aqueous acid solutions (8).

### 3.3 Solubility

Warfarin is soluble in acetone and dioxane. Moderately soluble in methanol, ethanol, isopropanol. Freely soluble in alkaline aqueous solutions (forms a water-soluble sodium salt). The sodium salt is freely soluble in water, alcohol, very slightly soluble in chloroform and ether (5). Practically insoluble in water, benzene, cyclohexane.

### 3.4 Alkalinity

A 1% solution has a pH of 7.2 to 8.3. (9).

### 3.5 Moisture Content and Hygroscopicity

Not more than 2%, determined by Fischer titration (9).

### 3.6 Dissociation Constant

pKa value is 5.0 (20°C) (9).

### 3.7 Storage

It should be stored in air-tight containers protected from light (4).

### 3.8 Spectral Properties

#### 3.8.1 Ultraviolet Spectrum

The ultraviolet spectrum of warfarin in 0.01 N sodium hydroxide, maximum at 308 nm (E 1%, 1 cm 462) (8).

Fig. 1, shows the U.V. spectrum of warfarin in methanol which was scanned from 200 to 400 nm. Using SP8-100 Pye Unicam UV-Vis spectrophotometer. It exhibits absorption maximum at 311 nm.

#### 3.8.2 Infrared Spectrum

The infrared spectrum of warfarin as KBr disc is



Fig. 1 Ultraviolet Spectrum of Warfarin in Methanol

presented in Fig. 2 and is recorded in Perkin-Elmer spectrophotometer model 580 B. The structural assignments have been correlated with band frequencies and are given the following table.

<u>Frequency (cm<sup>-1</sup>)</u>	<u>Assignment</u>
1518 ) 1600 )	C = C aromatic stretch
1645	C = C stretch
1710	C = O stretch
3390	OH stretch

Clarke (8) reported the principal peaks as KBr disc are 1517, 1599, 1640 cm<sup>-1</sup>.

Other characteristic finger print bands are 1450, 1420 and 1350 cm<sup>-1</sup>.

### 3.8.3 Mass Spectrum and Fragmentometry

The mass spectrum of warfarin is shown in Fig. 3. The spectrum was carried out on Finnigan MAT 1020 mass spectrometer. The sample was introduced by direct probe. The spectrum shows a molecular ion peak at m/e 308 and a base peak at m/e 103. The most prominent fragments, their relative intensities and possible structures are as follows:

<u>m/e</u>	<u>Relative intensity %</u>	<u>Fragment</u>
308	> 1	M <sup>+</sup>
103	100	$\overline{\text{C}_8\text{H}_7}^+$
43	50	$\overline{\text{CH}_3\text{CO}}^+$

Trager *et al* (7) have published detailed study on the use of mass spectroscopy for the analysis and identification of human metabolites of warfarin. The electron impact mass spectrum of

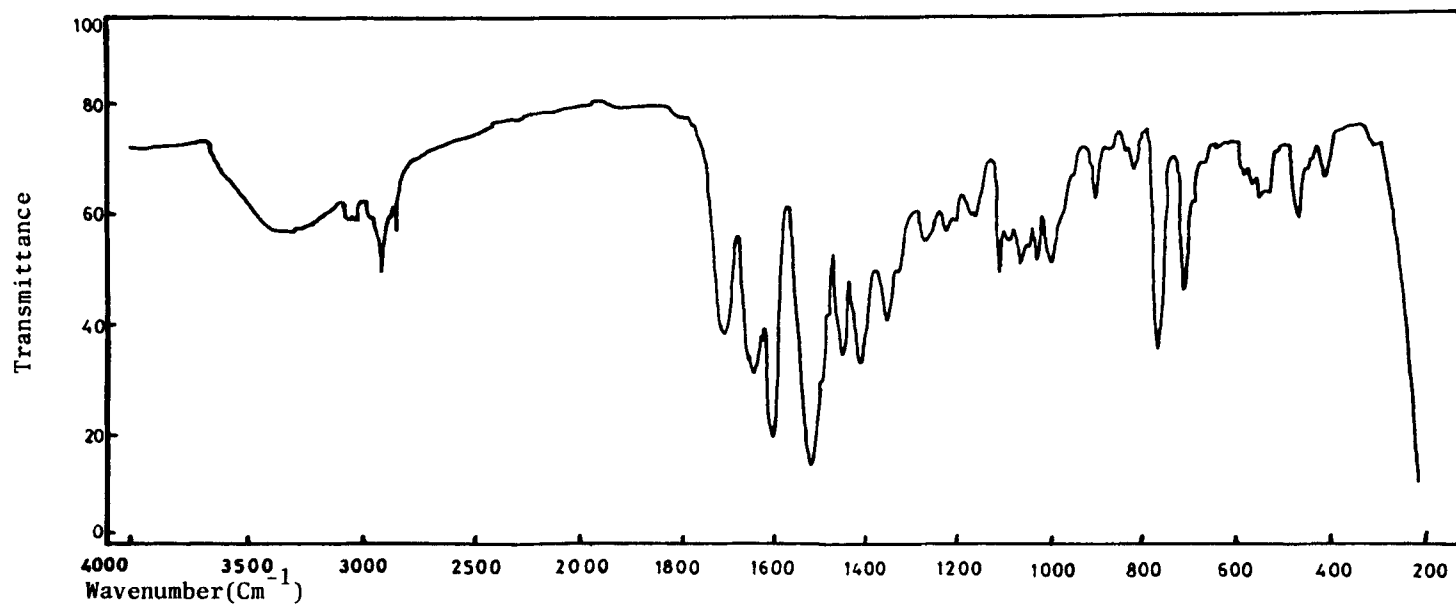


Fig. 2: Infrared Spectrum of Warfarin as KBr disc.

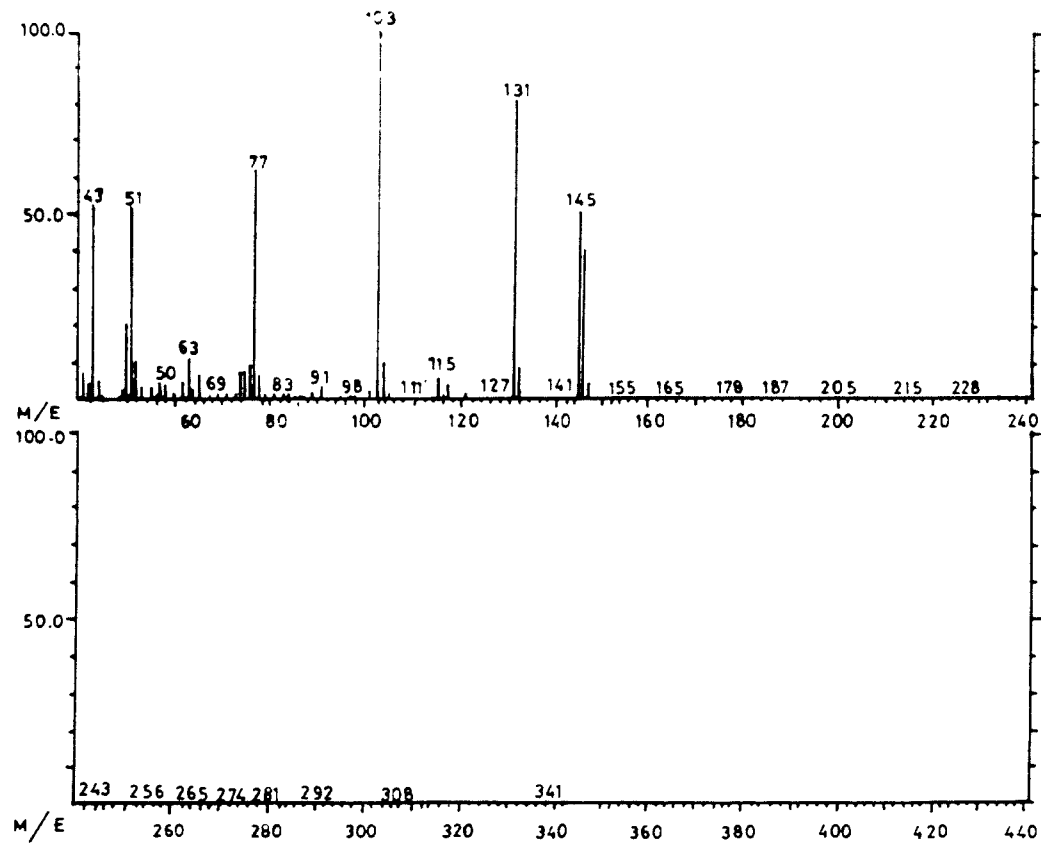
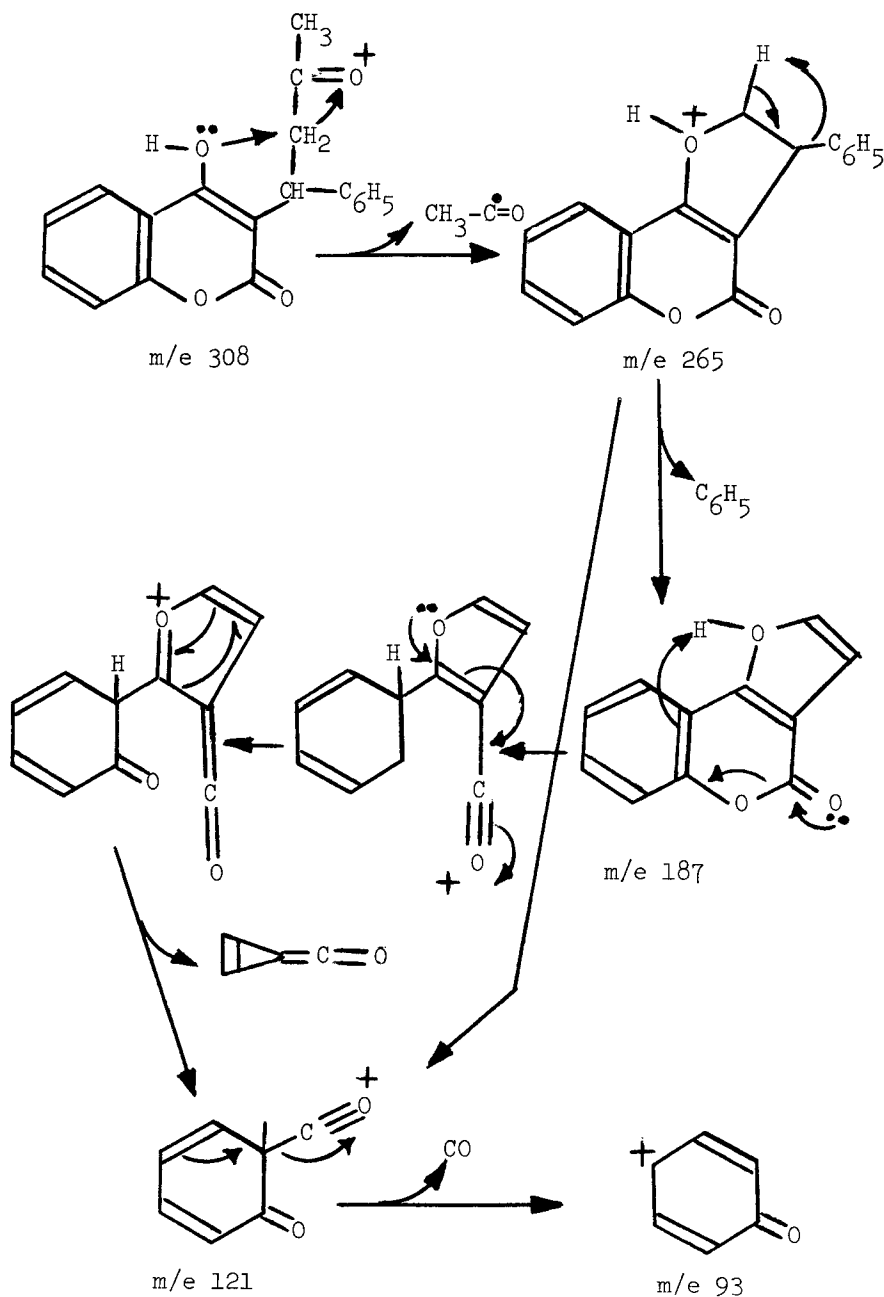


Fig. 3. Mass Spectrum of Warfarin

warfarin is reproduced in Fig. 3a, according to the above authors, the drug gives the following fragmentations:



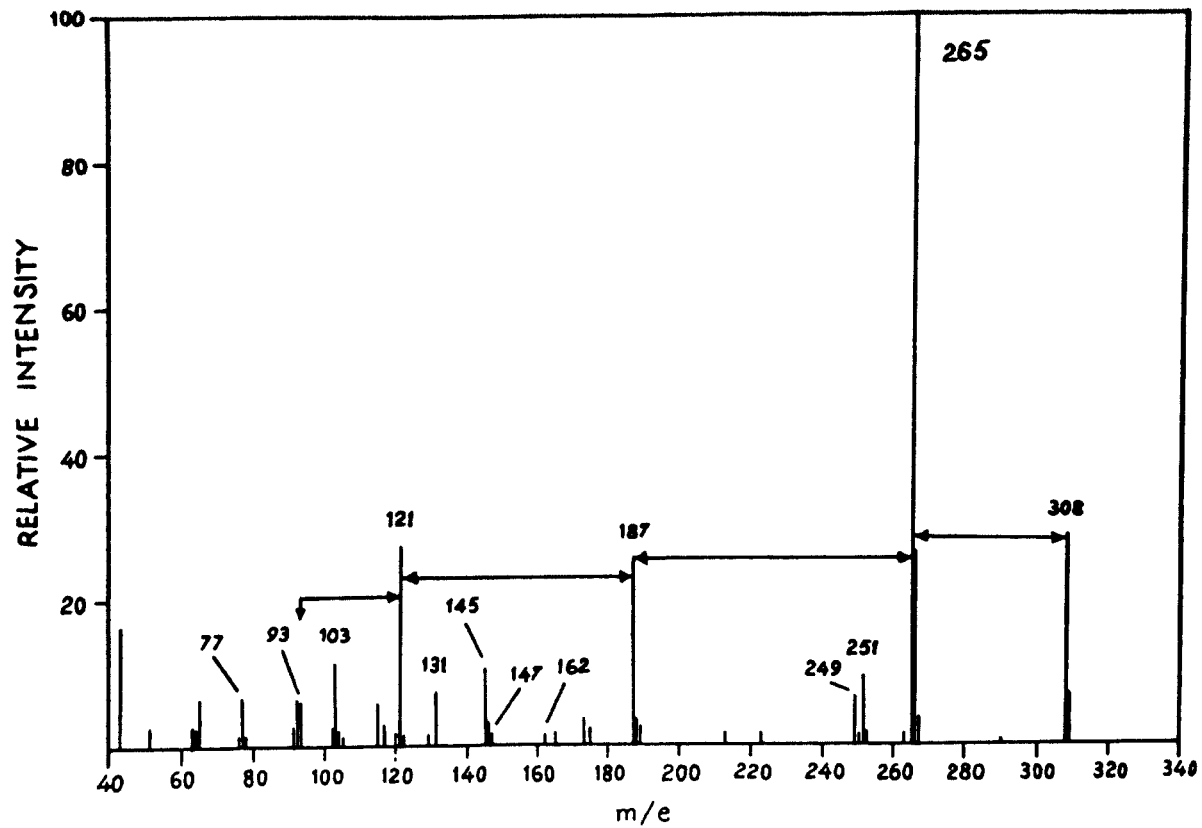


Fig 3<sub>a</sub> The (EI) Mass spectrum of warfarin (7)

### 3.8.4 Nuclear Magnetic Resonance Spectra

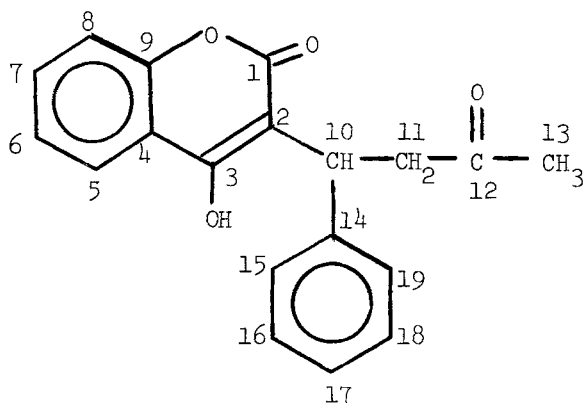
#### 3.8.4.1 Proton Spectrum

The PMR spectrum of warfarin in D<sub>2</sub>O + DSS (2,2 dimethyl-2-silapentane-5-sulfonate) was recorded on a Joel-FX-100 NMR spectrometer using DSS as a reference standard, Fig. 4. The structural assignments are shown below:

<u>Proton</u>	<u>Chemical shift</u>	<u>Multiplicity</u>
Aromatic protons	7-7.9	multiplet
$\text{-}\underset{ }{\text{CH}}\text{-}$	4.6	triplet
$\text{-}\underset{2}{\text{CH}_2}\text{-}\overset{\overset{\text{O}}{\parallel}}{\text{C}}\text{-}$	3.48	doublet
$\text{-}\underset{3}{\text{CH}_3}$	2.26	singlet

#### 3.8.4.2 Carbon-13 Spectrum

The noise-decoupled and off-resonance C-13 NMR spectra of warfarin are presented in Figs. 5 and 6 respectively. The spectra were obtained on Jeol-XL-100 NMR spectrometer. Spectral assignments are listed below:





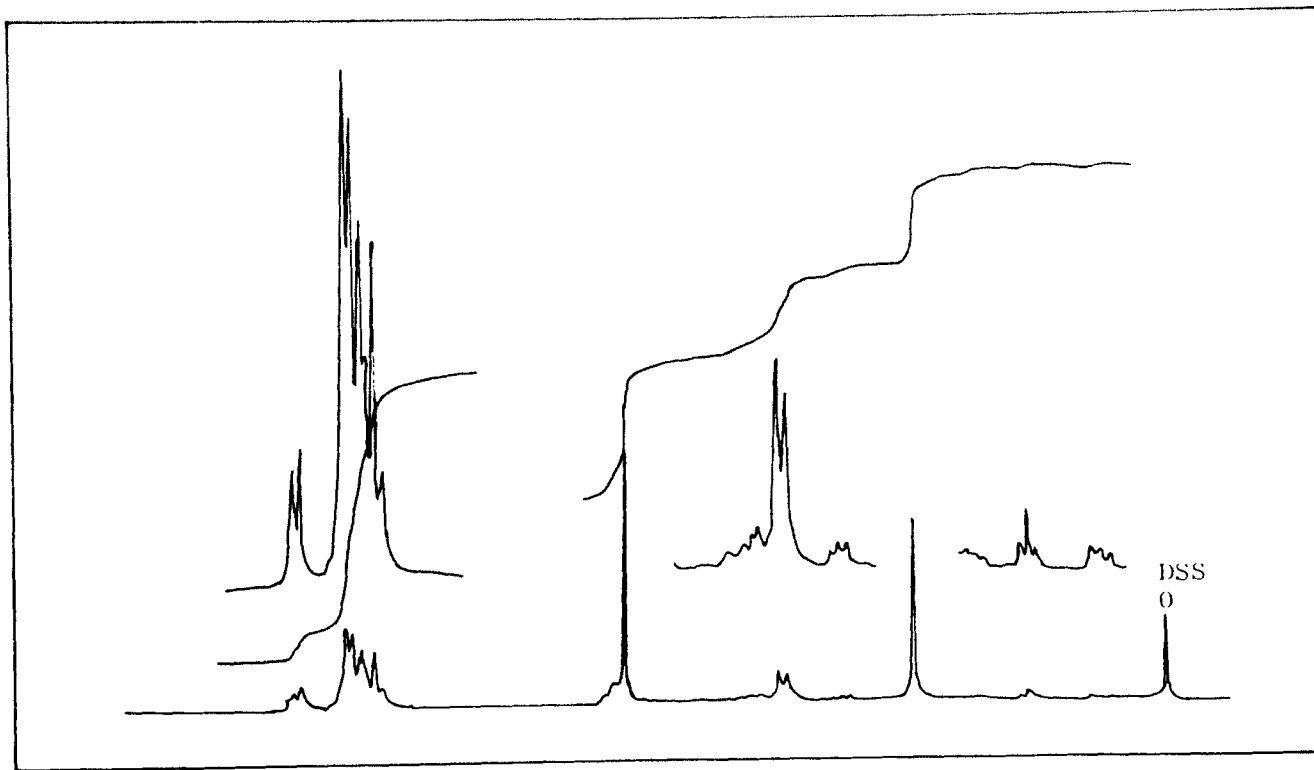


Fig. 4: Proton NMR Spectrum of Warfarin in D<sub>2</sub>O.

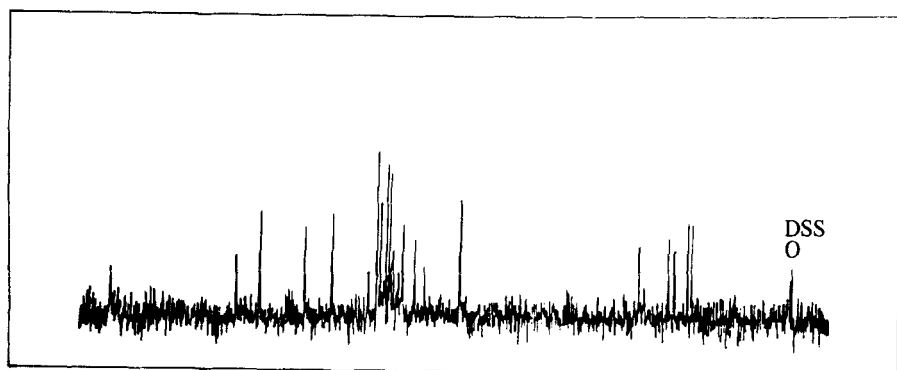


Fig. 6: Carbon-13 NMR Spectrum of Warfarin (Off-Resonance) in D<sub>2</sub>O.

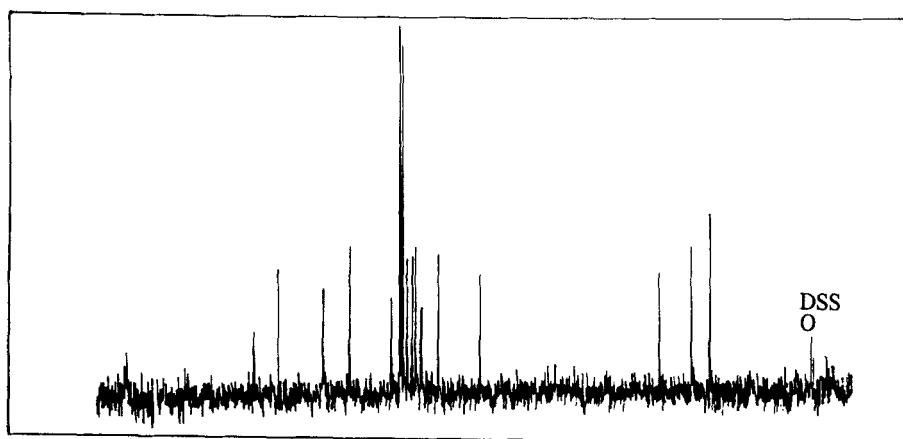


Fig. 5: Carbon-13 NMR Spectrum of Warfarin (Noise decoupled) in D<sub>2</sub>O.

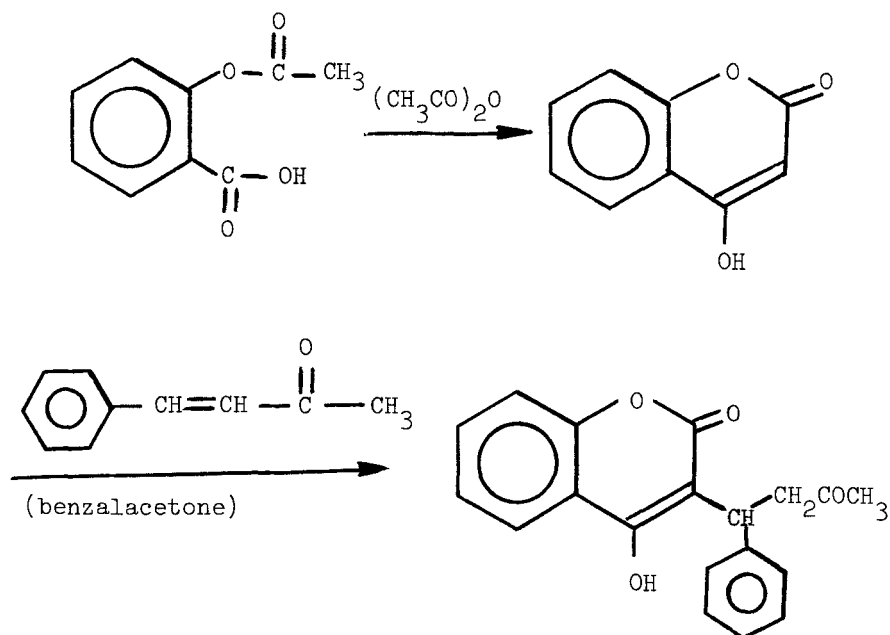
<u>Carbon No.</u>	<u>Chemical Shift (ppm) relative to DSS</u>	<u>Multiplicity</u>
1	177.77	singlet
2	146.70	singlet
3	169.90	singlet
4	105.38	singlet
5	128.51	doublet
6	124.11	doublet
7	125.90	doublet
8	118.60	doublet
9	155.4	singlet
10	37.92	doublet
11	48.25	triplet
12	218.60	singlet
13	32.05	quartet
14	133.68	singlet
15 and 19	130.86	doublet
16 and 18	129.86	doublet
17	126.87	doublet

The carbon-13 nuclear magnetic resonance spectroscopy of the anticoagulant warfarin and related compounds have also been published (10).

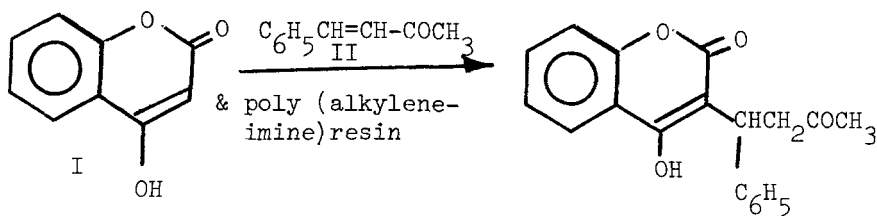
#### 4. Synthesis

Warfarin can be synthesized by the following methods.

- a) Acetylsalicylic acid, on treatment with acetic anhydride in presence of a sodium salt, followed by acidification gives the 4-hydroxy-coumarin. This, on reaction with benzal-acetone gives warfarin (11).

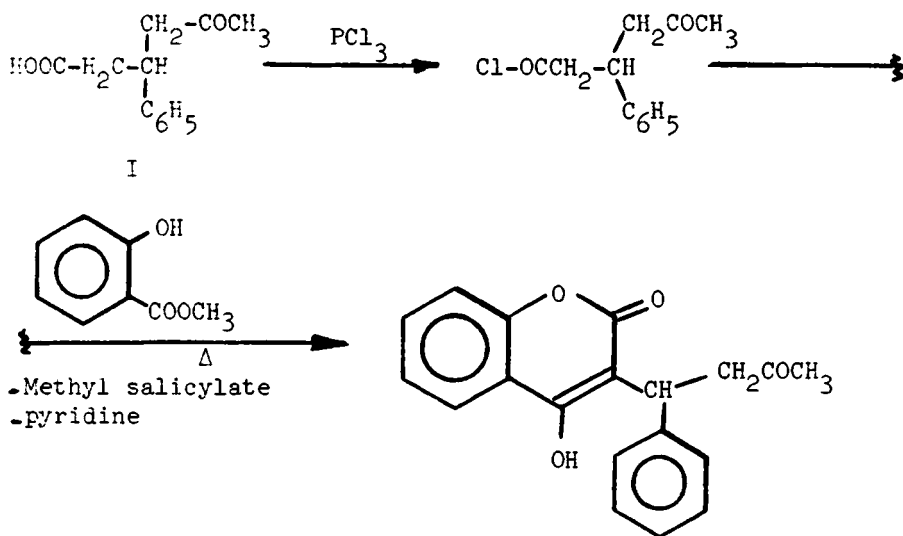


- b) Equivalent amounts of 4-hydroxycoumarin (I) and benzalacetone (II) are heated in presence of an ion exchanger, particularly a poly-(alkylenimine)-resin to give the 3-( $\alpha$ -phenyl- $\beta$ -acetyethyl) derivative of hydroxycoumarin (12). Thus a melt of 4-hydroxycoumarin (500 g) and 450 g of benzalacetone was passed at  $120^\circ$  through an



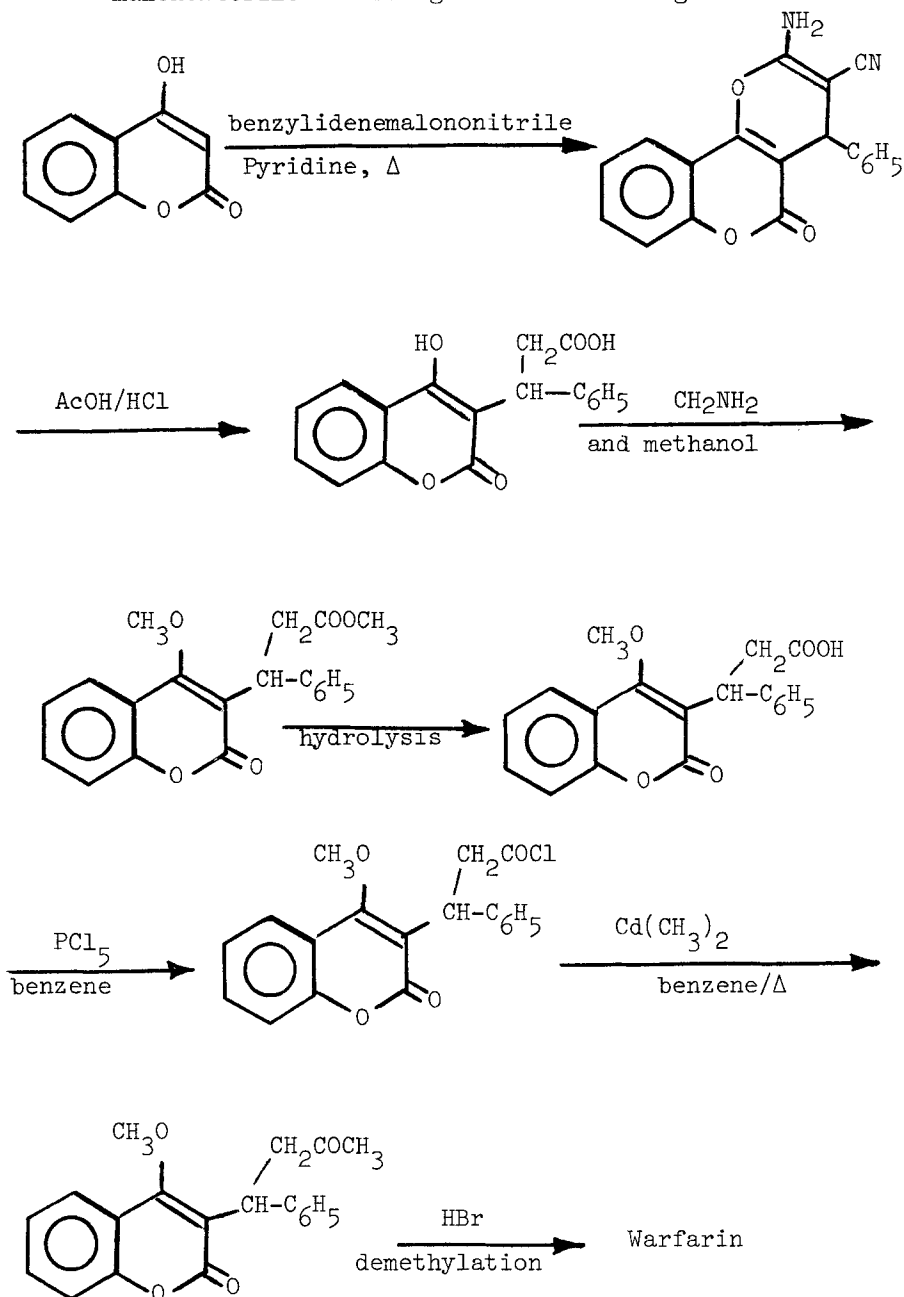
electrically heated column packed with a poly-(alkylenimine)-resin so that the reaction time was 30 minutes. The product m.p.  $150-5^{\circ}$  and the yield was 550 gm. The author has described another procedure that gives better yield and purer compound (m.p.  $161^{\circ}$  in 80% yield). Other routes for the synthesis of warfarin from 4-hydroxycoumarin and benzalacetone have been reported (13-17).

- c)  $\beta$ -phenyl- $\gamma$ -acetylbutyric acid (I) is heated with  $\text{PCl}_3$  at  $60-70^{\circ}\text{C}$ . The resulting acid chloride is dissolved in toluene and the solution is heated with methyl salicylate and filtered. A few drops of pyridine are added to the filtrate, boiled and evaporated. The resulting compound is extracted with acetone to give warfarin (18).



- d) Wiener et al (19) have described a new method for the synthesis of warfarin using an intermediate obtained from the reaction of 4-hydroxycoumarin with benzylidene-

malononitrile according to the following scheme:



## 5. Pharmacokinetics

### 5.1 Absorption, Fate and Excretion

The principal mode of administration of warfarin is by oral route, but sometimes it is administered intravenously and intramuscularly. Large initial dose ranging between 40-50 mg is necessary to establish the therapeutic plasma drug level. After oral administration, a measurable rise in plasma warfarin level is observed, only after 30-120 minutes ingestion. After this time gap the total absorption and the rate of absorption rise rapidly until a peak concentration is achieved in 6-9 hours. The rate of absorption is dose-dependent. Once in circulation, the drug is bound to plasma albumin to the extent of 99%, during long term therapy, which largely prevents its diffusion into red blood cells, cerebrospinal fluid, urine and breast milk (Orme et al (20)). Food decreases the rate, but not the extent of absorption of warfarin. Warfarin is eliminated in an exponential manner. The half-life of drug (41-45 hours) is essentially dependent of the original dose and the mode of administration and the volume of distribution is that of the albumin space, 11 to 12% of body weight (Wagner (21)). Considerable amount of metabolites are eliminated through kidney, but they do not account for the whole original dose. Very few amount of unchanged warfarin can be detected in urine.

In man, the dextro warfarin enantiomorph is metabolized by side chain reduction to a secondary alcohol. Whereas levo warfarin is metabolized by oxidation of the ring, primarily to 7-hydroxy warfarin (Lewis and Trager, (22)). These inactive metabolites products are to some extent conjugated with glucuronic acid, undergo an enterohepatic circulation and are ultimately excreted in the urine and stool.

## 6. Therapeutic and Other Uses

Warfarin is the most widely used coumarin anticoagulant and is often considered as a drug of choice. After an initial dose of 40-50 mg, a therapeutic prothrombin time is usually achieved in 36 hours and the effect lasts for a further period of 36 hours. If warfarin is employed for a long term anticoagulant therapy, it takes about three days for the prothrombin time to return to normal, after drug discontinuation. Warfarin has a unique property of being water soluble among the anti-prothrombin drugs and if vomiting prevents oral administration, it can be administered by intramuscular, intravenous or rectal route. It is the only oral anticoagulant available for parenteral administration. The drug

also has a mild bronchodilator activity and has shown to dilute coronary arteries and improve coronary blood flow (23). The inherent danger associated with the long action of warfarin is considerable cumulative effect of this drug.

## 7. Toxicity

The principal toxic effect of warfarin is haemorrhage. It depresses formation of prothrombin and increases capillary fragility leading to haemorrhages. Bleeding may occur from any side, particularly from mucous membranes, skin, the gastrointestinal and genito urinary tract. The major haemorrhage manifestation most frequently encounters in hematuria. It is usually not fatal or followed by impairment of renal function. Bleeding from the gastrointestinal tract is usually secondary to previously undetected peptic ulcers. It may occur with therapeutic doses and the prothrombin time within usual therapeutic range (5, 24). Single doses of warfarin, unless very large, do not usually exert a lethal action; in man, doses above 100 mg may be dangerous. Rats may survive single doses of 50 mg/kg, but they are killed by repeated dosing for 5 days with 1 mg/kg. Sheep and cattle will tolerate single doses of up to 50 mg/kg (8).

Since 1960, three apparent independent outbreaks of resistance to warfarin in rats have been reported; the resistance has been shown to be heritable and to be dependent on a single dominant autosomal gene (Greaves and Ayres, 1967) (8).

Toxicity of warfarin depends on repeated ingestion. Daily intake by man of 1-2 mg/kg for 6 days has produced severe illness in an attempted suicide. Haemorrhage is the main unwanted effect caused by warfarin therapy (3).

Warfarin itself is a highly toxic poison, but the fact that it is needed at such low concentrations in baits and that these must be eaten repeatedly to cause symptoms makes it less likely to injure pets and children than certain other poisons. It has had a good record of safety and is considered one of the less dangerous rat and mouse control material (25).

The major risk from warfarin therapy is of haemorrhage from almost any organ of the body. Other side-effects, occasionally reported include urticaria, dermatitis, alopecia, fever, nausea, vomiting, diarrhoea, hypersensitivity reactions and rarely, skin necrosis. Early signs of overdosage are mild bleeding from the gums or elsewhere and the presence of erythrocytes in the urinary deposit (26).



## 8. Methods of Analysis

### 8.1 Identification

#### a) Ultraviolet Spectroscopic Test

B.P. 1973 (4) and U.S.P. XIX (6) cite the use of ultraviolet absorption spectrum of warfarin in 0.01N sodium hydroxide as a means of identification comparing some characteristic absorption maxima of the drug. Maximum in case of warfarin is at 308 mμ.

#### b) Infrared Spectroscopic Test

B.P. 1973 (4) and U.S.P. 1980 (6) cite the use of infrared absorption spectrum of warfarin as a means of identification comparing some characteristic absorption band of the drug using potassium bromide disk, the principal peaks are 1517, 1599, and 1640 ( $\text{cm}^{-1}$ ) (8).

#### c) Melting Point Test

B.P. 1973 (4) also uses melting point of the drug as a means of identification. Dissolve 1 g of warfarin sodium in 25 ml of water, add 2 drops of dilute hydrochloric acid and filter. Melting point of precipitate after washing with water is about 162°C.

#### d) Clathrate Color Test

B.P. 1973 (4) describes a clathrate color test for the identification of warfarin. Dissolve 1 g in 10 ml of water, add 5 ml of nitric acid and filter. To the filtrate add 2 ml of N/10 potassium dichromate and shake for 5 minutes. The clathrate yields a light green-blue solution.

#### e) Thin-Layer Chromatographic Test

Lau-Cam and Chung-Fong (27) developed a thin-layer chromatographic method of identification of warfarin, which is of great interest to forensic toxicologist and the analytical chemists. TLC has proven particularly advantageous for routine work owing to simplicity. Separations were accomplished on silica gel G plates using the following solvent

mixture: Ether : chloroform : acetone (60:30:1). Spots may be clearly seen in ultraviolet light or by exposing chromatogram to iodine vapours for five minutes.

## 8.2 Spectrophotometric Methods

### 8.2.1 Ultraviolet Spectrometry

U.S.P. 1980 (6) has described a spectrophotometric method for the determination of warfarin in tablets. Weigh accurately a portion of the powder, equivalent to about 40 mg of warfarin sodium, and transfer to a 250 ml volumetric flask. Add 50 ml of sodium hydroxide solution (1 in 2500), shake for about 15 minutes, then add sodium hydroxide solution (1 in 2500) to volume. Filter through paper, discarding the first 20 ml of the filtrate. Pipet 10 ml of the subsequent filtrate into a 125 ml separator, add 2 ml of water, adjust with hydrochloric acid to a pH of less than 3, using short-range pH indicator paper, and mix. Extract with one 25 ml and two 15 ml portions of chloroform, filtering each through a pledget of glass wool into a 100 ml volumetric flask. Rinse the pledget, and add chloroform to volume. Dissolve an accurately weighed quantity of U.S.P. Warfarin Reference Standard, previously dried in vacuum over phosphorus pentoxide for 4 hours, in chloroform, and dilute quantitatively and stepwise with chloroform to obtain a standard solution having a known concentration of about 15  $\mu\text{g}$  per ml. Concomitantly determine the absorbances of both solutions in 1-cm at the wavelength of maximum absorbance at about 307 nm, with a suitable spectrophotometer, using chloroform as the blank. Calculate the quantity, in mg, of  $\text{C}_{19}\text{H}_{15}\text{NaO}_4$  in the portions of tablets taken by the formula  $2.5C(1.071 \text{ Au/As})$ , in which C is the concentration, in  $\mu\text{g}$  per ml, of USP Warfarin Reference Standard in the standard solution, 1.071 is the ratio of the molecular weight of warfarin sodium to that of warfarin, and Au and As are the absorbances of the solution from Warfarin Sodium Tablets and the Standard solutions, respectively.

O'Reilly et al (28) developed a reproducible and

sensitive spectrophotometric method for the determination of warfarin in plasma. To 4 ml plasma, add 2 ml of water and 1 ml of 3N hydrochloric acid. The contents were taken into a separating funnel containing 20 ml ethylenedichloride (EDC). The contents were shaken for 10 minutes and allowed to settle for 30 minutes. The EDC layer was taken in a second separator containing 5 ml of 0.1 M phosphate buffer (pH 7.2). After shaking for 4 minutes 15 ml of EDC layer was taken into a third separator containing 5 ml of 2.5 N sodium hydroxide, the mixture is again shaken for 4 minutes and alkaline aqueous phase containing warfarin was separated and centrifuged to separate residual EDC. The absorbance of aqueous phase is measured at 308 nm on spectrophotometer.

### 8.2.2 Fluorimetry

- a) Corn and Berberich (29) described a rapid, and simple assay for the determination of plasma warfarin. 10 ml of blood is collected in a tube containing 0.1 ml of sodium citrate (38%) or 20 mg of ethylene diamine tetraacetic acid (EDTA) or 14 mg of sodium oxalate (the later two anticoagulants are added as solution and then solutions are dried). Plasma is separated by centrifugation. 0.2 ml of plasma is mixed with 4 ml of acetone (Reagent grade) and centrifuged for 3 minutes, supernatant is separated and fluorescence is measured (designated as  $F_1$ ). Then 0.05 ml of 0.1 N HCl is added to the tube and the fluorescence is again measured (designated as  $F_2$ ). The excitation and emission wavelength for this study are 365  $\mu$  and 415  $\mu$  respectively, using acetone as a blank. A standard warfarin plasma prepared by adding 0.1 ml of warfarin (50  $\mu$ g/ml water) to 0.9 ml warfarin-free plasma, was carried through the procedure once daily. The decrease in the fluorescence caused by the addition of acid is used to determine warfarin concentration as follows:

$$\mu\text{g. warfarin/ml plasma} = \frac{5(F_1 \text{ unKn} - F_2 \text{ unKn})}{F_1 \text{ std.} - F_2 \text{ std.}}$$

- b) Bachmann (30) developed a rapid spectrofluorometric method for the determination of unbound warfarin in human plasma. According to this method 5 ml aliquotes of plasma are ultrafiltered through cellulose dialysis bags (19 mm diameter, 24A pore size) after addition of warfarin (2-8  $\mu\text{g/ml}$ ). Dialysis bags containing plasma are placed inside a conical support mounted in a 50 ml centrifuge tube. Samples are centrifuged at 754 X g at room temperature until 0.5 ml or 1.0 ml of ultrafiltrate could be collected. The extent of warfarin sequestration by the dialysis membrane was estimated by placing 5.0 ml of isotonic phosphate buffer (pH 7.4) containing warfarin inside the dialysis bag, and measured the warfarin concentration in the ultrafiltrate fluoremetrically using an excitation wavelength of 365 nm and emission of 415 nm.

### 8.3 Chromatographic Methods

#### 8.3.1 Gas Chromatography

Clarke (8) describes three GC systems:

- a) Column: 1% SE-30 on 100-120 mesh Anakrom ABS. 6 ft X 4 mm internal diameter borosilicate glass column. Column temperature : 180°, carrier gas: Argon , Gas flow: 65 ml per min at 180°, Detector: Argon ionisation detector or flame ionisation detector. Retention time 0.28 relative to diphenhydramine.
- b) Column: 3% QF-1 on 100-120 mesh Anakrom ABS, column temperature: 200°, carrier gas: Argon, gas flow: 80 ml per minute. Retention time 0.78 relative to diphenhydramine.
- c) Column: 10% Apiezon L on 80-100 mesh Chromosorb W-AWHMDS, column temperature: 210°, carrier gas: Argon or nitrogen. Gas flow: 50 ml per minute. Detector: flame ionisation detector, hydrogen 50 ml per minute, air flow 300 ml per minute. Retention time 0.78 relative to barbitone.

Midha *et al* (31) described a sensitive method for the determination of plasma warfarin, using gas chromatography. To a 2 ml plasma sample, add 2 ml of 3 N HCl and 20 ml of ethylene dichloride and 1 ml of phenylbutazone (5 µg/ml). The samples are extracted for 10 minutes on a flat bed shaker. After centrifugation at 3000 rpm for 10 minutes, the aqueous layer is removed by aspiration and 18 ml of ethylene dichloride is transferred to another tube containing 10 ml of 1 N NaOH. The extraction is repeated with 20 ml of ethylene dichloride and extract is evaporated to dryness at 85°C. The residue is redissolved in 25 µl of carbon disulphide and 2-3 µl of this solution is injected in gas chromatograph equipped with flame-ionization detector. The column is a spiral glass tube 1.8 meter long and 0.25 cm id, packed with 5% phenyl methyl dimethyl silicon (80-100 mesh). Nitrogen was used as a carrier gas at a flow rate of 63 ml/minute and the temperature was maintained between 260-280°C. Calibration curve was used to determine the concentration of warfarin.

### 8.3.2 Gas Liquid Chromatography

Kaiser and Martin (32) developed a gas chromatographic method for determination of warfarin in biological fluids using electron-capture-detector. 1 ml of plasma was transferred in a tube containing 0.1 ml of 3-( $\alpha$ -acetonyl-p-chlorobenzyl)-4-hydroxy-coumarin (as internal standard). Add 0.5 ml of aqueous sodium hydroxide (0.01 N) mix well. Add 0.5 ml, 3N sulphuric acid and 10 ml of ethylene dichloride. The mixture is shaken horizontally for 10 minutes and centrifuged. Dry 8 ml of ethylene chloride layer with a gentle stream of nitrogen. Dissolve the residue in 100 µl of ethylene chloride. Spot the sample on thin layer silica gel plate. Ascendingly develop each plate for a distance of 165 mm, air dry all chromatographs thoroughly at room temperature and visualize the zone corresponding to warfarin in UV lamp. Scrape the zone and extract it with 2 ml of methanol. Dry the methanol layer using nitrogen gas. Make the solution in hexane and inject 1 µl in gas chromatograph, having U shaped glass column (0.61 m X 3 mm i.d.) packed with 1% w/w OV-17 on Gas Chrom Q(80-100 mesh). The

column, injection port, and electron-capture-detector are maintained isothermally at 240, 275 and 285° respectively. Flow rates of nitrogen gas used as carrier and purge are maintained at 75 and 30 ml/minute respectively. Values for unknown concentration of warfarin are calculated using calibration curve.

The recovery of 98.8 + 10.9% are observed using this method.

### 8.3.3 High Pressure Liquid Chromatograph

- a) Bjornsson et al (33) developed a rapid sensitive and specific high pressure liquid chromatographic method for quantitative analysis of warfarin in plasma. The method involves a single solvent extraction of warfarin and the internal standard from acidified plasma, followed by evaporation and HPLC analysis. Concentration between 0.1 and 4.0 µg/ml can be measured with a coefficient of variation of 2.5%. Known metabolite of warfarin do not interfere with the analysis. Sample is prepared by taking 0.2 - 1 ml plasma in a tube containing 100 µg of para chlorowarfarin as internal standard. To this 0.5 ml of 0.5 N aqueous sulphuric acid and 5 ml of ether were added. The samples are extracted by mixing gently for 10 minutes, followed by centrifugation at 2500 rpm for 10 minutes. The organic phase is separated and dried at 40-50°C. The residue is dissolved in 25 µl of methanol for chromatographic analysis. 15-25 cm long, 6.3 mm od, 2.2 mm id reverse-phase column. Detection is done using variable wavelength UV-visible detector with 10 µl flow cell. The flow rate of the solvent mixture is fixed at 60 ml/hr with a column input pressure of 390-420 kg/cm. The peak height ratio of warfarin to the internal standard is plotted against amount of warfarin added.
- b) Robinson et al (34) described a HPLC method for quantitative analysis of warfarin in plasma. The method involves an extraction from acidified plasma, removal of basic substances and

re-extraction into ether. 2.5 ml of citrated plasma 0.5 ml of 1.5 N  $\text{H}_2\text{SO}_4$  and 5 ml of ether are added. The tubes are shaken for 5 minutes and centrifuged. The ether layer is separated and extracted with 2 ml of 2N  $\text{H}_2\text{SO}_4$  and re-extracted with 5 ml of ether. Ether layer is separated and dried. The residue is dissolved in 0.1 ml methanol and 10  $\mu\text{l}$  was injected in chromatograph with a variable wavelength detector, using C<sub>18</sub> Bondapak column, absorbance was measured at 308 nm. The mobile phase is 70:30 mixture of methanol and 0.5% acetic acid with a flow rate of 1 ml/minute. Concentration was determined using a standard curve. The method is sufficiently sensitive to measure low warfarin level and is linear to 9  $\mu\text{g/ml}$  and free from interference by common drugs.

- c) Mundy *et al* (35) developed a method for the analysis of warfarin in animal tissues, stomach contents, body fluids and feeding stuffs by HPLC. An adsorption column is used giving a lower limit of determination of about 0.025 ppm. 10 gm of sample is macerated with anhydrous sodium sulphate (10 gm) and chloroform (30 ml). The extract filtered through sintered glass and the residue triturated with further 15 ml of chloroform. The combined filtrates are concentrated to 10 ml under stream of nitrogen and extracted with 2 X 10 ml of aqueous sodium pyrophosphate solution (1% w/v). The aqueous extract is washed with chloroform (5 ml), acidified with hydrochloric acid (2.2 ml of 5N solution and extracted with chloroform (3 X 10 ml). The chloroform layer is dried and residue is dissolved in 0.1 ml of chloroform. 5-10  $\mu\text{l}$  of this solution is injected in chromatograph. The elute being isopropinol-iso-octane (2:98) and the pressure 60 kg/cm<sup>2</sup> giving a flow rate of 1 ml/minute. The absorbance is measured at 270 nm using UV detector.
- d) Perez (36) described a simple method for the determination of warfarin in rodenticide preparation. 20 gm of bait is extracted with 150

ml of Dimethyl formamide (DMF) for 1 hr. using a mechanical shaker. The mixture was filtered and filtrate is made 200 ml with DMF. 25  $\mu$ l of the filtrate was injected in the chromatograph with a flow rate of 2 ml/min. The reverse phase column was eluted with 0.005 M pentane sulphonic acid in methanol: water (16:40) and UV-visible detector set at 280 nm. The concentration of warfarin was determined comparing the mean heights of the peak of standard and the test material. The recovery of warfarin was about 97.8%.

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# NALOXONE HYDROCHLORIDE

Mahmoud M. A. Hassan,  
Mohamed E. Mohamed and  
Mohammad Saleem Mian

*King Saud University  
Riyadh, Saudi Arabia.*

1. Description	454
1.1 Nomenclature	454
1.2 Formulae	454
1.3 Molecular Weight	455
1.4 Elemental Composition	456
1.5 Appearance, Color, Odor, and Taste	456
2. Physical Properties	456
2.1 Melting Range	456
2.2 Solubility	456
2.3 Optical Rotation	456
2.4 Crystal Structure	456
2.5 Spectral Properties	458
3. Synthesis	469
4. Disposition of Naloxone Hydrochloride	477
5. Metabolism	477
6. Methods of Analysis	479
6.1 Identification	479
6.2 Titrimetric Methods	479
6.3 Chromatographic Analysis	480
References	487

# 1. Description

## 1.1 Nomenclature

### 1.1.1 Chemical Names (1-5)

- (a) Morphinan-6-one-4,5-epoxy-3,14-dihydroxy-17-(2-propenyl)-, hydrochloride, (5 $\alpha$ ).
- (b) 17-Allyl-4,5 -epoxy-3,14-dihydroxy-morphinan-6-one hydrochloride.
- (c) 1-N-allyl-7,8-dihydro-14-hydroxynormorphinone hydrochloride.
- (d) 12-Allyl-7,7a,8,9-tetrahydro-3,7a-dihydroxy-4a H-8, 9C-imino-ethanophenanthro [4,5-bcd] furan-5(6H)-one hydrochloride.
- (e) 1-N-allyl-14-hydroxynordihydromorphinone.
- (f) N-allylnoroxymorphone hydrochloride.
- (g) (-)-N-Allyl-4,5-epoxy-3,14-dihydroxy-6-oxomorphinan hydrochloride.
- (h) Allylnoroxymorphone hydrochloride.

### 1.1.2 Generic Name

Naloxone hydrochloride.

### 1.1.3 Trade Names

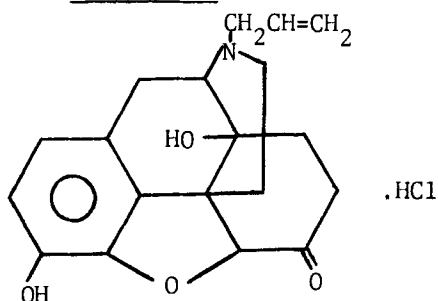
Narcan-Neonatal, EN-1530, Narcan, Narcon.

## 1.2 Formulae

### 1.2.1 Empirical

Anhydrous  $C_{19}H_{22}ClNO_4$ .

Dihydrate  $C_{19}H_{22}ClNO_4 \cdot 2H_2O$ .

1.2.2 Structural1.2.3 Stereochemistry

The numbering is the standard numbering for morphine (which follows that for phenanthrene). Naloxone differs structurally from oxymorphone, only by substitution of an allyl group for the N-methyl group. In addition it differs from morphine by a keto group in stead of an H atom at position 14, and by saturation of the 7,8 double bond. While morphine possesses asymmetric centers at positions 5,6,9,13 and 14, position 6 in naloxone (the keto position) is not asymmetric. The 5,6,7,8,13,14 ring in naloxone adopts the boat conformation, while this ring in morphine has the chair conformation. Otherwise, the absolute configurations of naloxone and morphine are believed to be the same (6,7). Model of naloxone is shown in Fig. A.

1.2.4 CAS Registry Number

Anhydrous [357-08-4]

Dihydrate [51481-60-8]

1.3 Molecular Weight

Anhydrous 363.84

Dihydrate 399.87

#### 1.4 Elemental Composition

C, 69.70%; H, 6.47%; N, 4.28%; O, 19.55%.

#### 1.5 Appearance, Color, Odour and Taste

A white to slightly off-white powder; odorless; taste bitter.

### 2. Physical Properties

#### 2.1 Melting Range

200° to 205°.

#### 2.2 Solubility

Soluble in water, in dilute acids, and in strong alkalis; slightly soluble in alcohol; practically insoluble in chloroform and in ether. Aqueous solutions are acidic. An 8.07% solution in water is isotonic with serum.

#### 2.3 Optical Rotation

$[\alpha]_D$  - 170 to - 181 calculated on the dried basis, determined in a solution containing 25 mg per ml.

#### 2.4 Crystal Structure

The crystal structure of naloxone hydrochloride dihydrate was determined by Sime et al (8). Naloxone hydrochloride dihydrate ( $C_{19}H_{21}NO_4 \cdot HCl \cdot 2H_2O$ ) is orthorhombic, space group  $P2_12_12_1$ , with  $a = 7.833[3]$ ,  $b = 13.185[5]$ ,  $c = 18.569[5] \text{ \AA}$ ,  $Z = 4$ . The structure was refined to a weighted  $R_2$  of 0.084 ( $R = 0.091$ ) for 1156 observed reflections. The structure of naloxone is similar to that of morphine. The two  $H_2O$  molecules are involved in hydrogen bonding with naloxone and  $Cl^-$ . Naloxone hydrochloride dihydrate forms colourless sturdy square prisms. The systematic extinctions,  $h00, h \text{ odd}; 0k0, k \text{ odd}; 00l, l \text{ odd}$  indicate unambiguously space group  $P2_12_12_1$ . The density, measured by flotation in  $CCl_4/C_6H_6$ , is  $1.35 \text{ g cm}^{-3}$ ; the calculated density is  $1.385 \text{ g cm}^{-3}$  for  $Z = 4$  ( $C_{19}H_{21}NO_4 \cdot HCl \cdot 2H_2O$ ). A stereo view (8) of the cation is shown in Fig. B. The numbering is the standard numbering for morphine. Bond lengths and

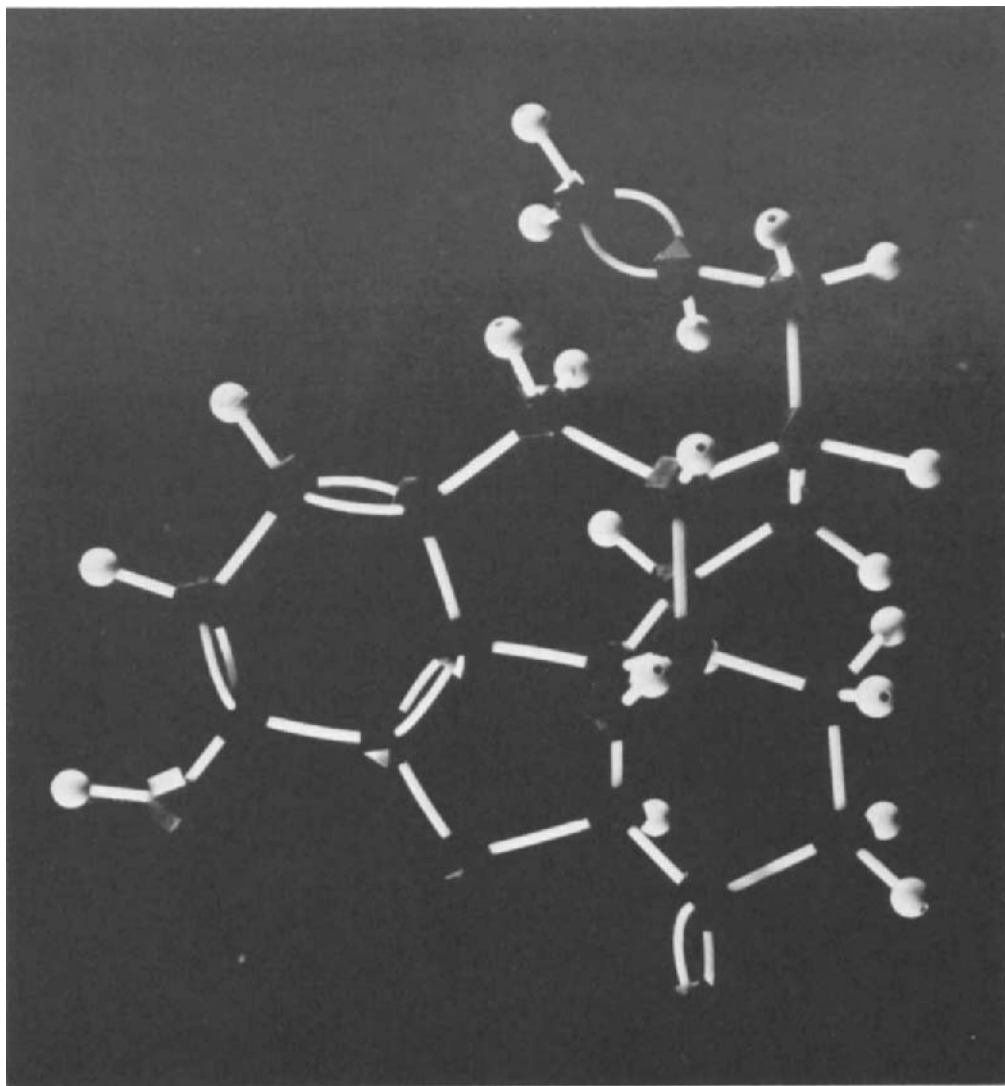


Fig. A Model of Naloxone



angles are shown in Tables 1 and 2. Packing of the naloxone cation and  $\text{Cl}^-$  anion is determined primarily by hydrogen bonding. A suggested scheme is shown in Fig. C. Both OH groups, the  $\text{N}^+\text{H}$ , the two water molecules appear to be involved for a total of seven hydrogen bonds per asymmetric unit (Table 3). The quaternary N atom has its H atom directed towards the  $\text{Cl}^-$  ion;  $\text{NH}^+ \dots \text{Cl}^-$  is  $3.14 \text{ \AA}$ , compared with  $3.07 \text{ \AA}$  in cocaine hydrochloride (9) and an average of  $3.21 \text{ \AA}$  (10). This bond and three intramolecular bonds of the N atom are nearly tetrahedrally arranged. In addition  $\text{Cl}^-$  is surrounded by three more hydrogen bonds, one to the OH group at position 6 ( $2.9 \text{ \AA}$ ), and the other two to water molecules:  $\text{Cl}^- \dots \text{H-O}[5] = 3.14$ ,  $\text{Cl}^- \dots \text{H-O}[6] = 3.15 \text{ \AA}$ . Three of the hydrogen bonds,  $\text{O}[1] \dots \text{Cl}$ ,  $\text{Cl} \dots \text{O}[5]$  and  $\text{O}[5] \dots \text{O}[3]$  are in nine membered rings, in the cavities of which lie the allyl groups. This is somewhat similar to the hydrogen bonding in valinomycin (11) in which two hydrogen bonds are in 13-membered rings, into the cavities of which are directed two free carbonyl groups. It should be noted that naloxone is capable of forming a variety of hydrogen bonds, not only with itself but with water molecules. It may be that bridging water molecules, hydrogen bonded between drug and receptor, play an important role at the receptor site.

## 2.5 Spectral Properties

### 2.5.1 Infrared Spectrum

The IR spectrum of naloxone hydrochloride as KBr-disc is shown in Fig. 1. The KBr-disc was recorded on a Perkin-Elmer 580 B Infrared spectrometer. The structural assignments have been correlated with the following band frequencies (Table 4).

Table 1. Interatomic Distances ( $\text{\AA}^\circ$ ).

O(1)-C(3)	1.363	C(8)-C(7)	1.527
C(3)-C(2)	1.416	C(7)-C(6)	1.520
C(2)-C(1)	1.380	C(6)-C(5)	1.588
C(1)-C(11)	1.482	C(6)-O(3)	1.156
C(11)-C(12)	1.327	C(14)-C(9)	1.549
C(12)-C(4)	1.343	C(9)-C(10)	1.607
C(4)-C(3)	1.332	C(10)-C(11)	1.496
C(2)-C(4)	1.377	C(13)-C(15)	1.537
O(2)-C(5)	1.455	C(15)-C(16)	1.498
C(5)-C(13)	1.527	N — C(16)	1.534
C(13)-C(12)	1.538	N — C(9)	1.446
C(13)-C(14)	1.498	N — C(17)	1.568
O(4)-C(14)	1.372	C(17)-C(18)	1.412
C(14)-C(8)	1.553	C(18)-C(19)	1.277

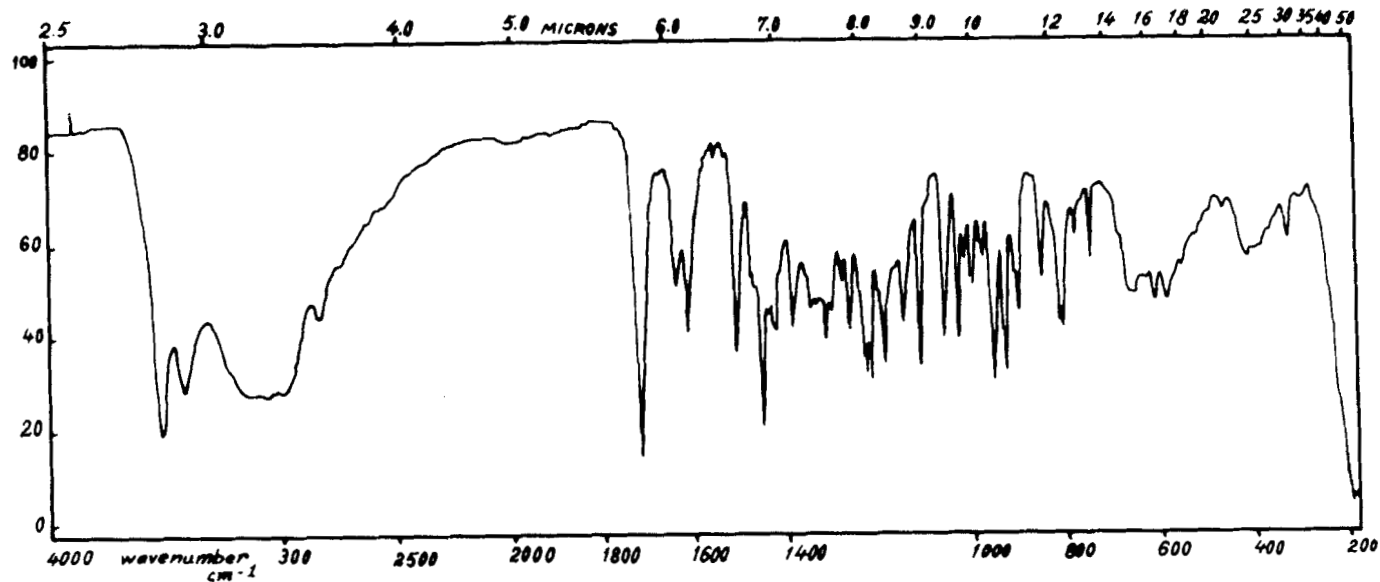
Table 2. Bond Angles ( $^\circ$ ).

O(1)-C(3)-C(4)	127.5	C(12)-C(13)-C(15)	110.1
O(1)-C(3)-C(2)	115.9	C(13)-C(14)-C(8)	110.9
C(4)-C(3)-C(2)	116.3	C(13)-C(14)-C(9)	107.1
C(3)-C(2)-C(1)	123.0	C(8)-C(14)-C(9)	110.5
C(2)-C(1)-C(11)	117.6	C(8)-C(14)-O(4)	109.2
C(1)-C(11)-C(12)	114.5	C(13)-C(14)-O(4)	110.6
C(10)-C(11)-C(1)	120.8	C(9)-C(14)-O(4)	108.5
C(11)-C(12)-C(4)	126.7	C(13)-C(15)-C(16)	110.5
C(13)-C(12)-C(11)	123.5	C(15)-C(16)-N	109.3
C(13)-C(12)-C(4)	109.8	C(16)-N — C(9)	112.4
C(12)-C(4)-C(3)	121.7	C(16)-N — C(17)	111.2
C(12)-C(4)-O(2)	111.3	C(9)-N — C(17)	114.0
C(3)-C(4)-O(2)	127.0	N — C(9)-C(14)	106.3
C(4)-O(2)-C(5)	105.8	N — C(9)-C(10)	114.2
O(2)-C(5)-C(13)	105.3	C(9)-C(10)-C(11)	110.4
C(6)-C(5)-C(13)	113.7	C(5)-C(6)-O(3)	118.4
C(6)-C(5)-O(2)	106.8	C(7)-C(6)-O(3)	127.7
C(5)-C(13)-C(12)	97.3	C(5)-C(6)-C(7)	113.6
C(5)-C(13)-C(15)	111.1	C(6)-C(7)-C(8)	112.4
C(12)-C(13)-C(14)	110.3	C(7)-C(8)-C(14)	107.5
C(15)-C(13)-C(14)	108.3	N — C(17)-C(18)	111.6
C(5)-C(13)-C(14)	119.2	C(17)-C(18)-C(19)	125.7

Table 3. Interatomic Distances and Angles Involving Possible Hydrogen Bonds.

Type	Bond	Distance
Hydroxyl	O(1)-H...Cl <sup>-</sup>	2.971 $\text{\AA}^\circ$
	O(4)-H...O(6)	3.236
Quaternary amine	N <sup>+</sup> -H ....Cl <sup>-</sup>	3.144
Hydrate water	H-O(5)-H...O(3)	2.901
	H-O(5)-H...Cl <sup>-</sup>	3.136
	H-O(6)-H...Cl <sup>-</sup>	3.148
	H-O(6)-H...O(1)	2.813





*Fig.1 IR Spectrum of Naloxone Hydrochloride*

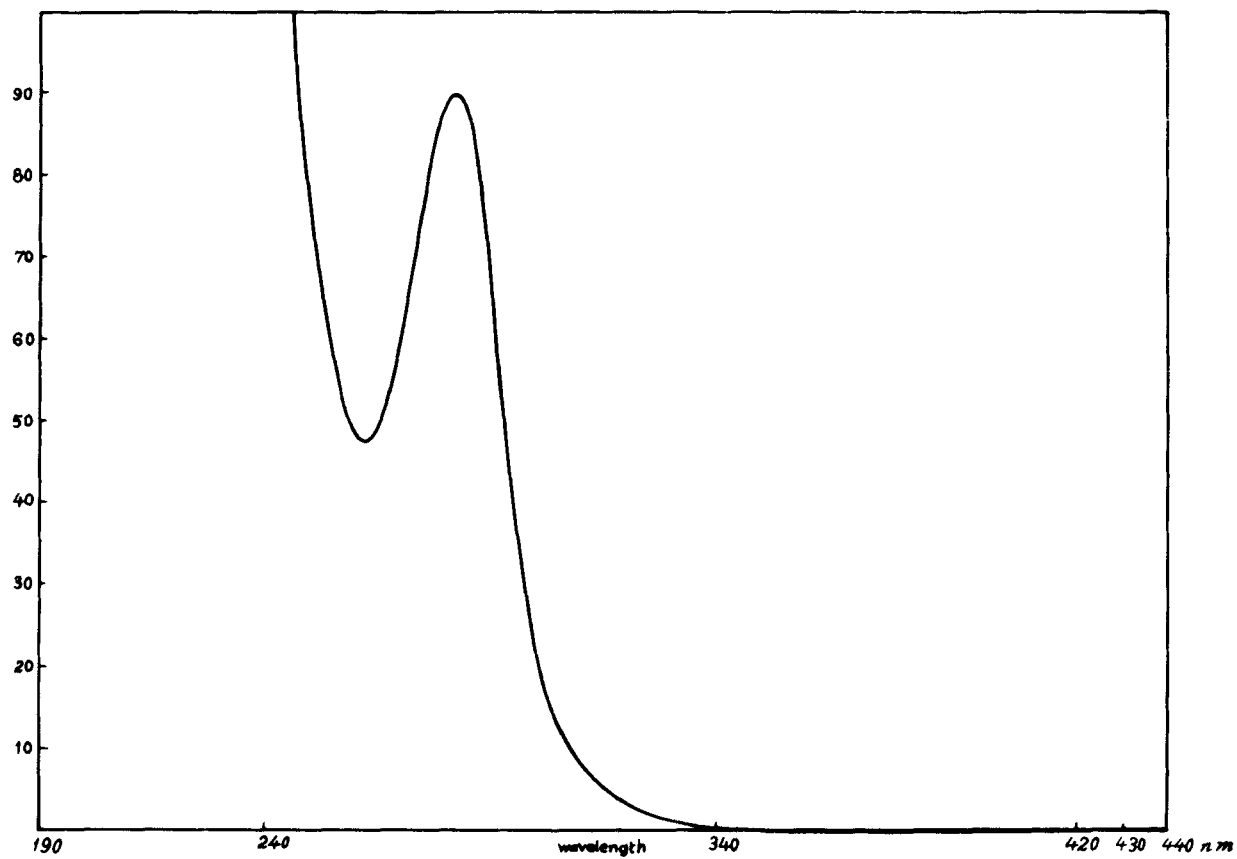
Table 4. IR Characteristics of Naloxone Hydrochloride.

<u>Frequency <math>\text{cm}^{-1}</math></u>	<u>Assignment</u>
3518	Free -OH and bonded OH
3420 ) 3260-2900)	Bonded OH and $\text{H}_2\text{O}$ of crystallisation.
2824	$\text{NH}^+$
1715	$\text{C}=\text{O}$
1640	$\text{C}=\text{C}$
1614	$\text{C}=\text{C}$ (aromatic)
1505	$\text{C}=\text{C}$ (aromatic)
1230, 1220	$\text{C}-\text{O}-\text{C}$
1189 1120	$\text{C}=\text{O}$
1030-1060	$\text{C}-\text{O}$ stretching
980, 925	$\text{CH}=\text{CH}_2$
850	Two adjacent aromatic hydrogens.

Other characteristic bands are 1450, 1420, 1382, 1265, 1149, 995, 900, 805, 812, 780 and  $745 \text{ cm}^{-1}$ .

#### 2.5.2 Ultraviolet Spectrum

The UV spectrum of naloxone hydrochloride in methanol was scanned from 440-190 nm on a Varian DMS-90 is shown in Fig. 2. The UV absorption curve shows a maximum at 288 nm and a minimum at 268 nm. Naloxone hydrochloride in 0.1 N hydrochloric acid shows a maximum at 281 nm and a minimum at 262 nm (12).



*Fig.2 UV Spectrum of Naloxone Hydrochloride in Methanol*

### 2.5.3 Nuclear Magnetic Resonance Spectra

#### 2.5.3.1 <sup>1</sup>H-NMR Spectrum

The PMR spectrum of naloxone hydrochloride in deuterated dimethylsulfoxide (DMSO D<sub>6</sub>) was recorded on a Varian T60A 60 MHz NMR spectrometer using tetramethylsilane (TMS) as an internal reference (Fig. 3). The following structural assignments have been made (Table 5).

Table 5. Characteristics of Naloxone Hydrochloride.

<u>Group</u>	<u>Chemical Shift (ppm)</u>	
H-1, H-2 (Aromatics)	6.7	(ABq)
3-OH, 14-OH	8.87	(bs)
H-5	5.03	(s)
CH=CH <sub>2</sub>	5.36-5.93	(m)
7,8,9,10,15,16-CH <sub>2</sub>	1.36-3.86	(m)

---

s = singlet, bs = broad singlet, q = quartet, m = multiplet.

#### 2.5.3.2 <sup>13</sup>C-NMR Spectra

<sup>13</sup>C-NMR completely decoupled and off-resonance spectra are shown in Fig. 4 and Fig. 5, respectively. Both were recorded over 5000 Hz range in deuterium oxide (conc. 100 mg/ml D<sub>2</sub>O) on FT-80A-80A-80 MHz NMR spectrometer, using 10 mm sample tube and dioxane as a reference standard at 20°C. The carbon chemical shifts are assigned on the basis of the chemical shift theory, additivity rules and the off-resonance splitting pattern (Table-6).

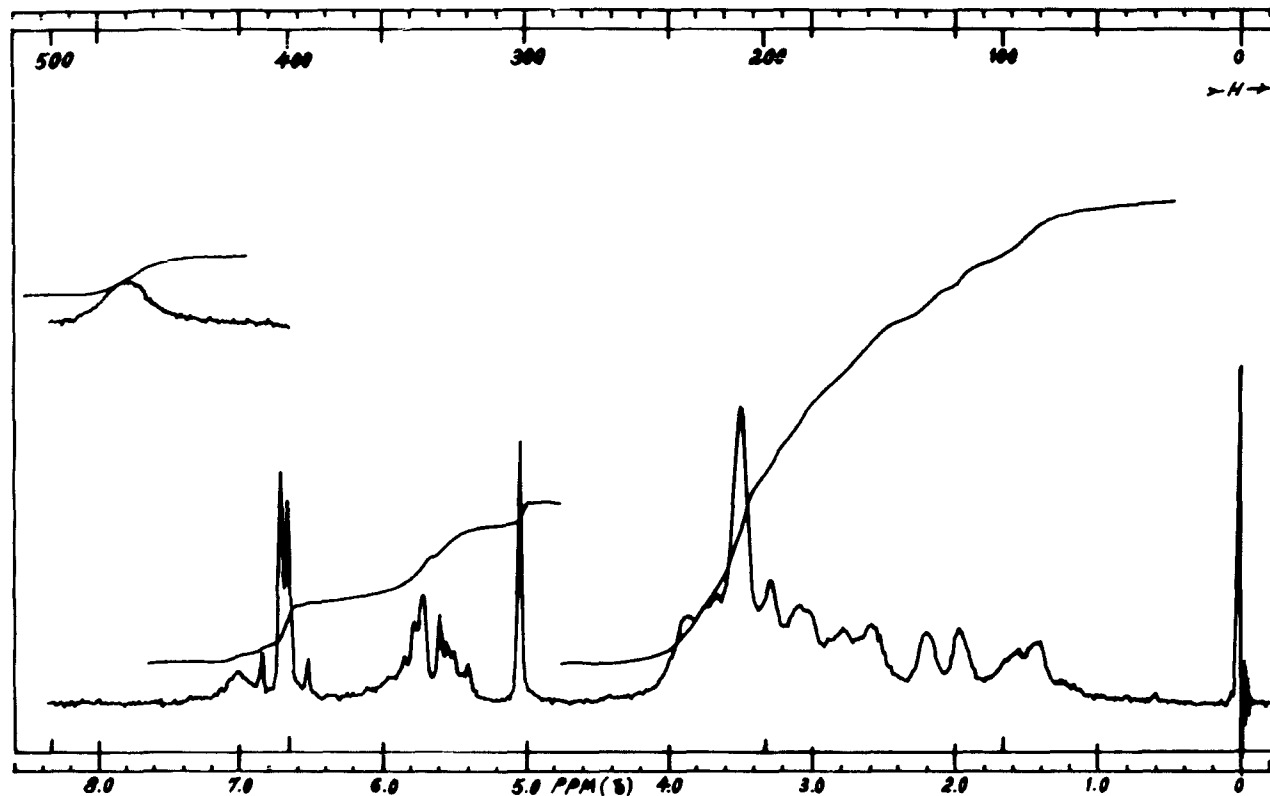


Fig.3 PMR Spectrum of Naloxone Hydrochloride in DMSO D<sub>6</sub>



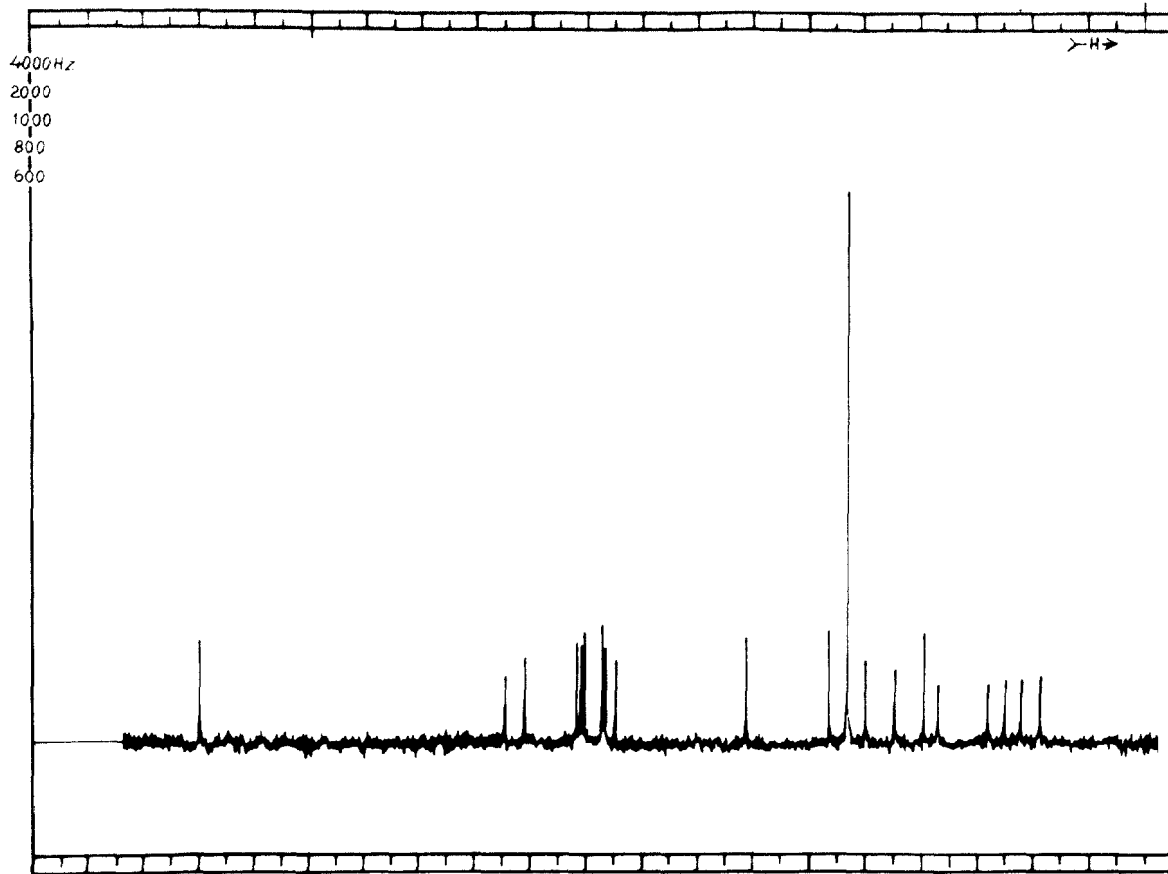


Fig. 4  $^{13}\text{C}$ -NMR Noise-Decoupled Spectrum of Naloxone Hydrochloride in  $\text{D}_2\text{O}$

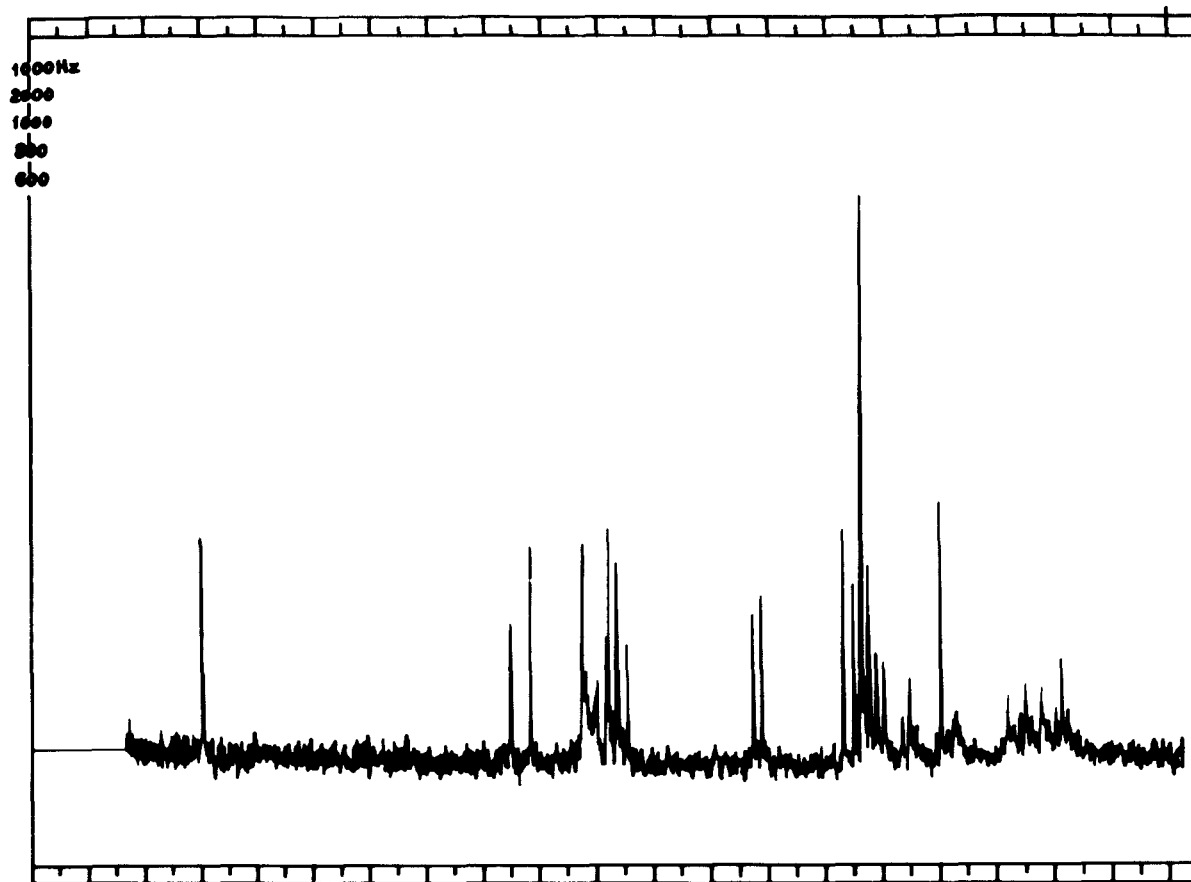


Fig. 5  $^{13}\text{C}$ -NMR off-Resonance Spectrum of Naloxone Hydrochloride in  $\text{D}_2\text{O}$

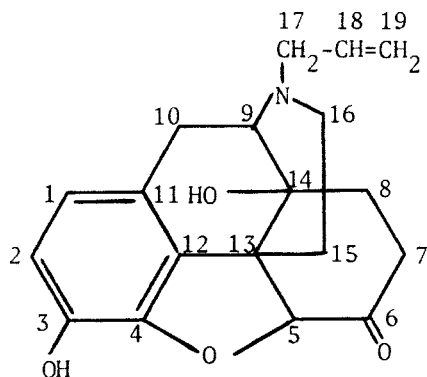


Table 6. Carbon Chemical Shifts of Naloxone Hydrochloride.

Carbon No.	Chemical Shift (ppm)	Carbon No.	Chemical Shift (ppm)
C-1	119.57 (d)	C-11	122.60 (s)
C-2	(d)	C-12	128.16 (s)
C-3	139.71 (s)	C-13	50.01 (s)
C-4	144.02 (s)	C-14	71.39 (s)
C-5	90.04 (d)	C-15	27.91 (t)
C-6	212.44 (s)	C-16	46.85 (t)
C-7	35.37 (t)	C-17	56.66 (t)
C-8	31.50 (t)	C-18	(d)
C-9	63.23 (d)	C-19	(t)
C-10	23.67 (t)		

---

s = singlet; d = doublet; t = triplet.

Carroll et al has also reported C-13 nuclear magnetic resonance spectra of morphine alkaloids (13).

#### 2.5.4 Mass Spectrum

The mass spectrum of naloxone hydrochloride is obtained by electron impact ionization and shown in Fig. 6. It was recorded on Finigan-Mat 1020 GC/mass spectrometer. The spectrum was scanned from 40 up to 350 a.m.u. Electron energy was 70 eV. The mass spectral data are shown in Table 7.

Table 7. The Most Prominent Fragments of Naloxone Hydrochloride.

<u>m/e</u>	<u>Relative intensity</u>	<u>Fragment</u>
70	100	Base peak (M <sup>+</sup> )
327	83.0	
83	70.0	
96	69.0	
55	62.0	
242	34.0	
201	28.0	
187	23.0	
286	20.0	

### 3. Synthesis

The synthesis of naloxone have been achieved according to the following three Schemes:

#### Scheme I (14)

- 1a : R = methyl, R' = H (oxymorphone)
- 1b : R = Allyl, R' = H (Naloxone)
- 1c : R = Methyl, R' = Acetyl
- 1d : R = Cyano, R' = Acetyl
- 1e : R = R' = H (noroxymorphone)
- 1f : R = VOC, R' = Acetyl
- 1g : R = H.HCl, R' = Acetyl

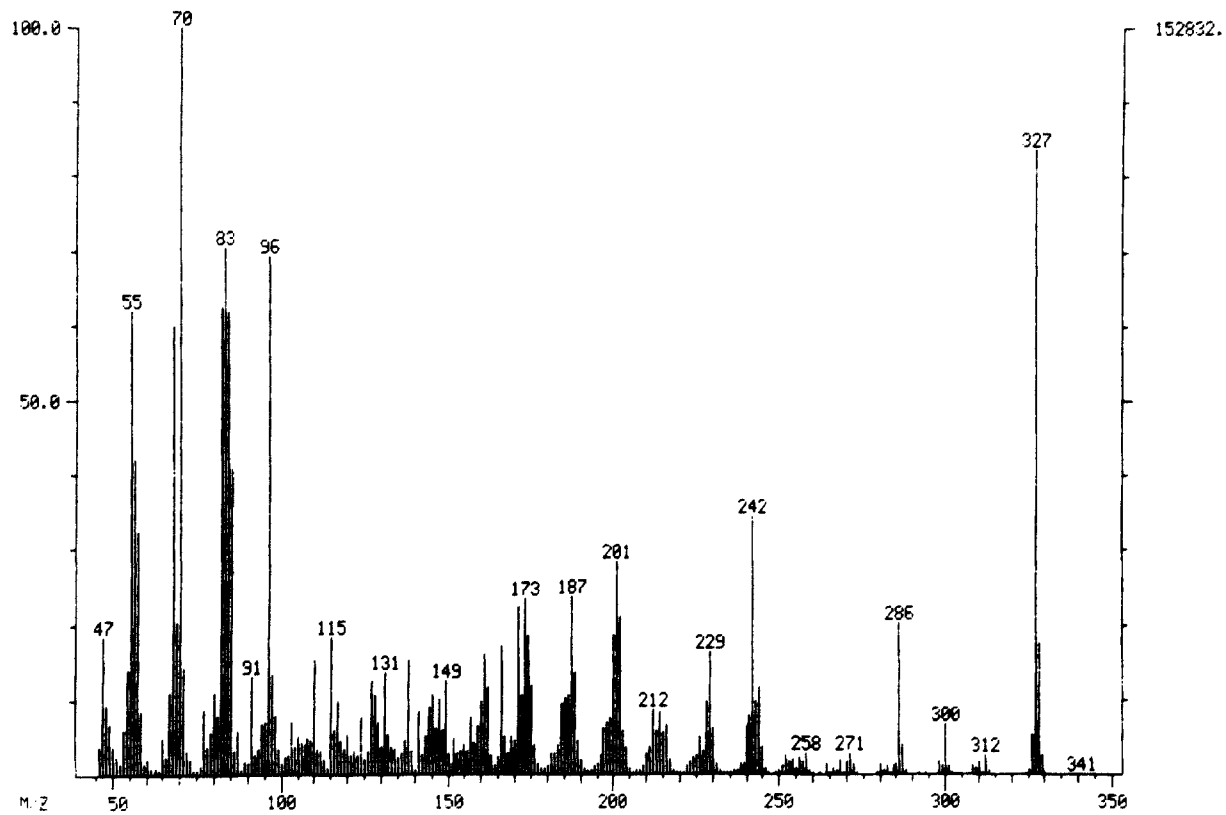
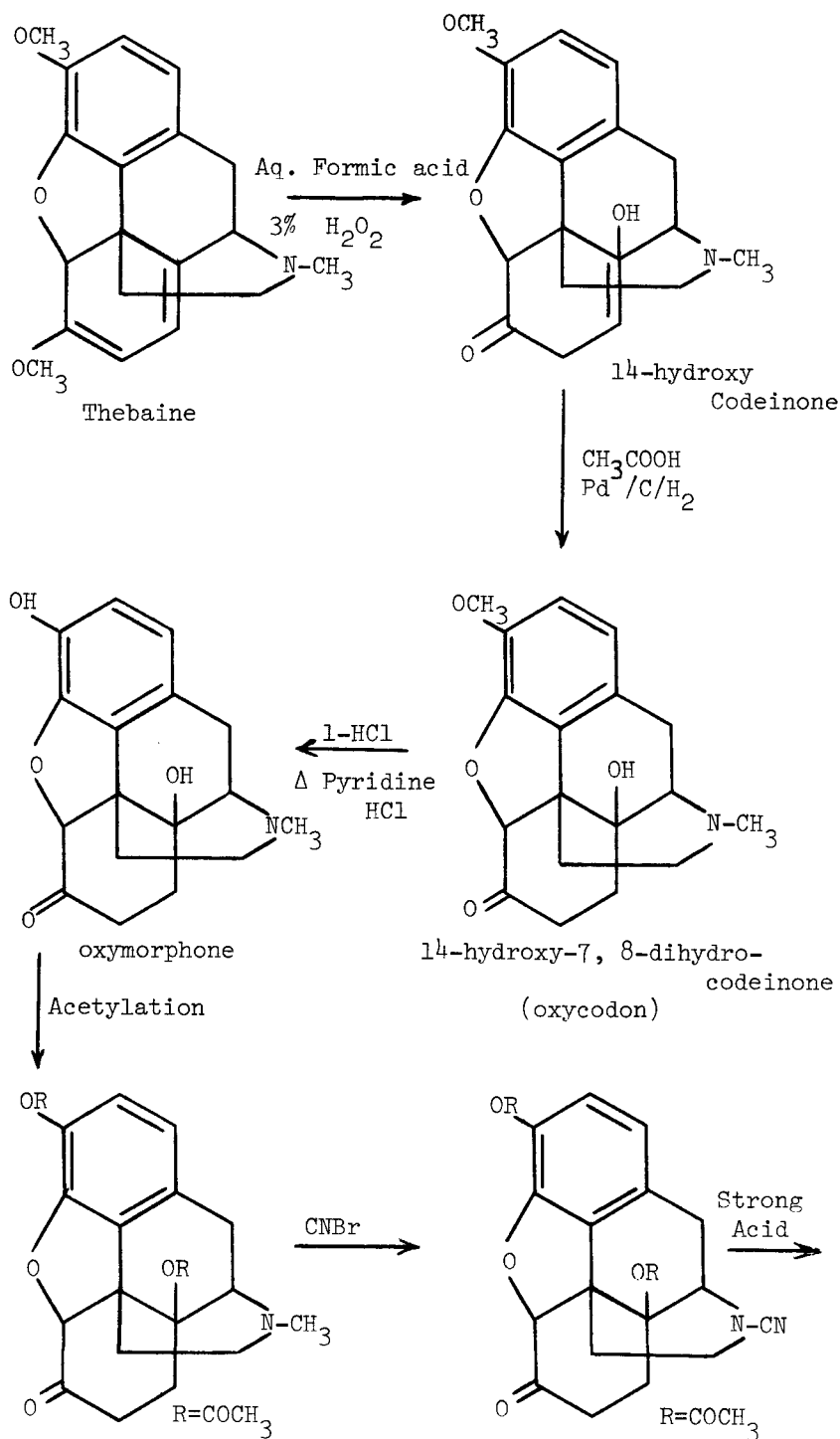
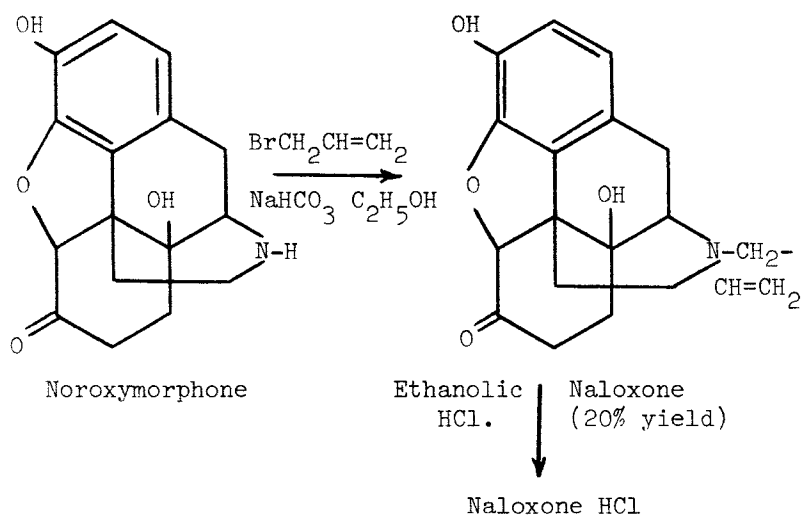


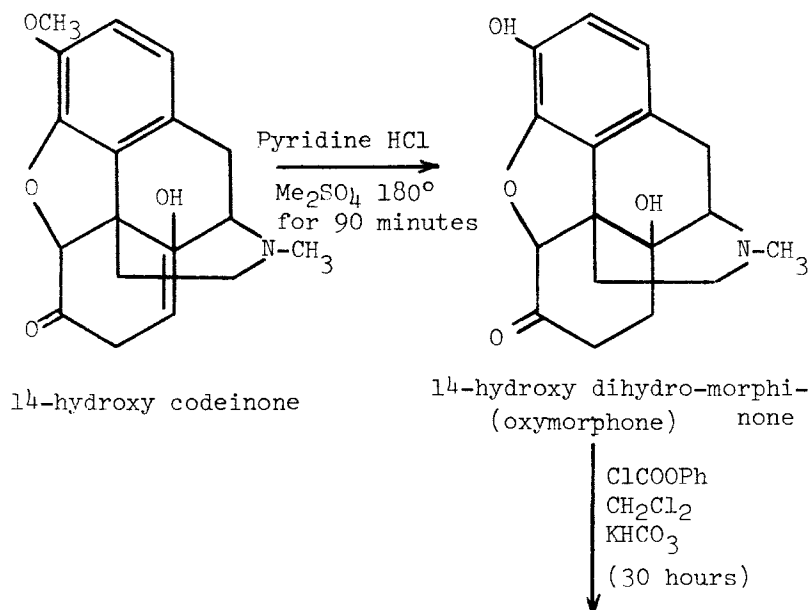
FIG. 6 EI-MASS SPECTRUM OF NALOXONE HYDROCHLORIDE.

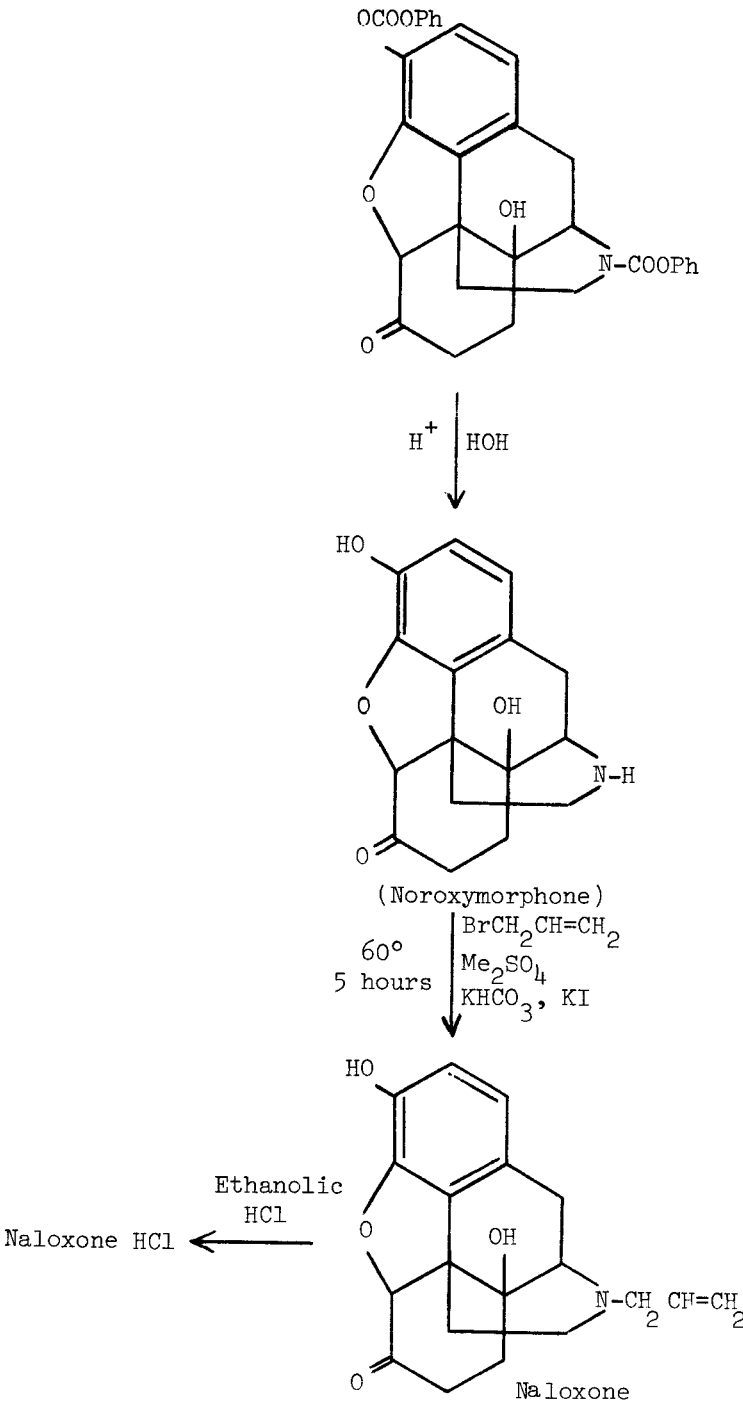
Scheme I.





Scheme II







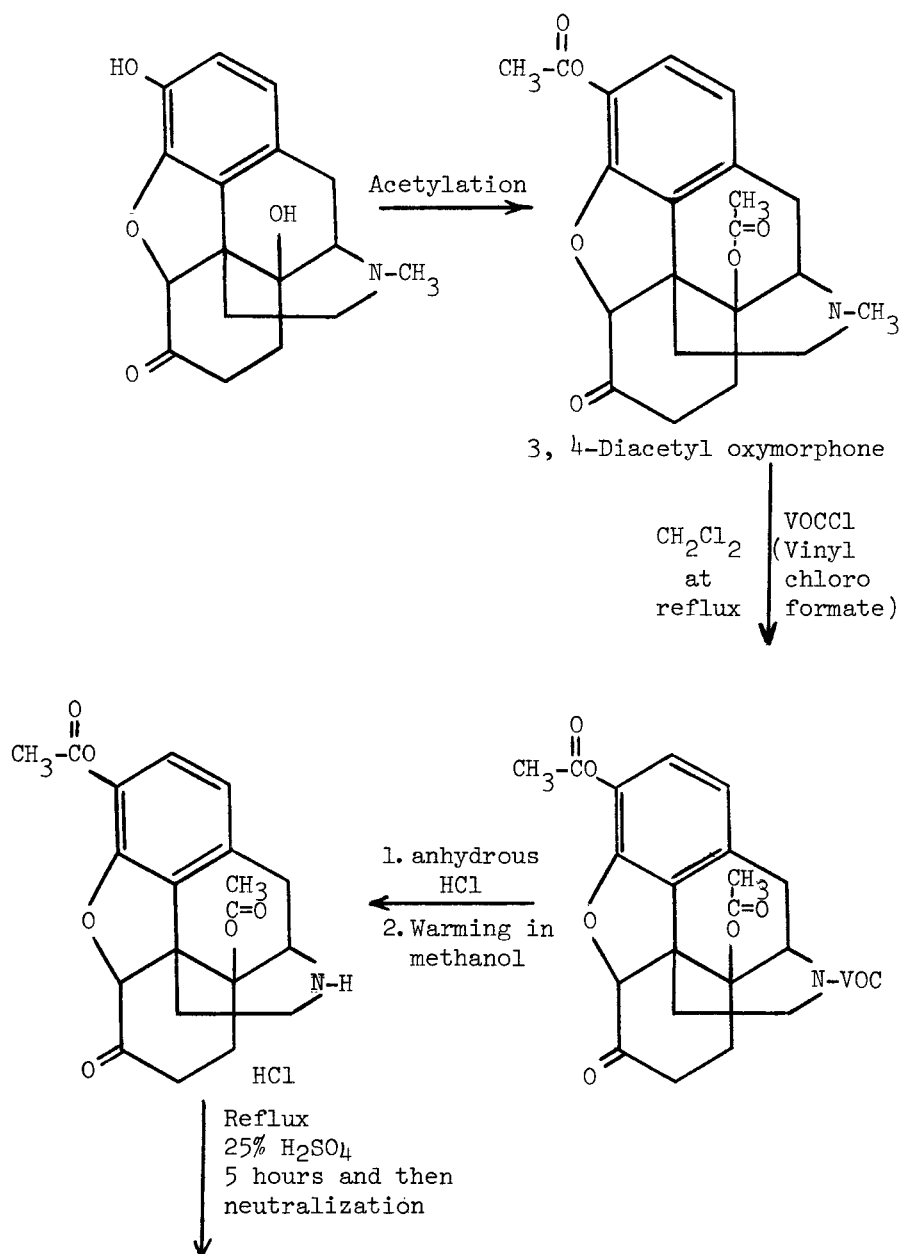
This work involves the preparation of oxymorphone (1a). Thebaine is dissolved in aqueous formic acid and treated with 30%  $\text{H}_2\text{O}_2$  after which neutralisation with aqueous ammonia yields 14-hydroxycodeinone. This is then dissolved in acetic acid and hydrogenated using Pd/C as a catalyst, to form 14-hydroxy-7,8-dihydrocodeinone (oxycodone). Oxycodone hydrochloride is then demethylated by heating with pyridine hydrochloride to yield crude oxymorphone hydrochloride which is then purified (15). Diacetylation of 1a to give 1c which is the N-demethylated with cyanogen bromide to afford the N-cyano compound (1d). Strong acid hydrolysis of the latter yields noroxymorphone (1e) which is converted to naloxone (1b) on treatment with allyl bromide. The published overall yield of crude naloxone from oxymorphone is only 20%.

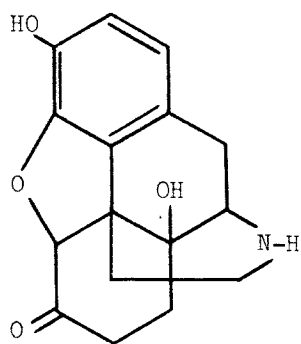
#### Scheme II (16)

14-Hydroxy codeinone was treated with pyridine hydrochloride in dimethylsulfate at  $180^\circ\text{C}$  for 90 minutes to give 14-hydroxy dihydromorphinone. Oxymorphone was treated with phenylchloroformate ( $\text{ClCO}_2\text{Ph}$ ) in methylene chloride containing potassium bicarbonate for 30 hours and the resulting product was hydrolysed to give demethyloxymorphone. The treatment of demethyloxymorphone by allyl bromide in dimethylsulphate containing potassium bicarbonate and potassium iodide warmed to  $60^\circ$  for 4-5 hours gave naloxone. Treatment with ethanolic hydrochloric acid gave naloxone hydrochloride.

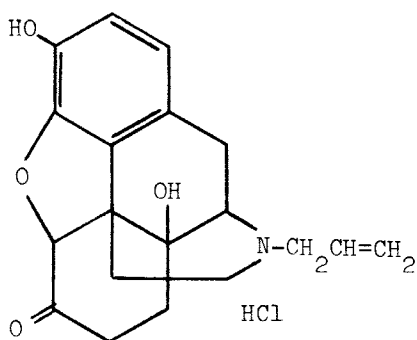
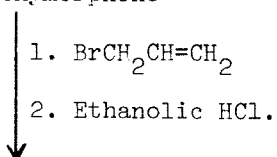
#### Scheme III (17)

When 3,14-diacetyloxymorphone (1c) was N-demethylated with vinylchloroformate ( $\text{VOCCl}$ ) in dichloroethane at reflux, the N-VOC compound (1f) was obtained in essentially quantitative crude yield. The VOC and acetyl groups were most efficiently removed by a three step process in which anhydrous  $\text{HCl}$  was first bubbled through  $\text{CH}_2\text{Cl}_2$  solution of (1f) to produce a normal adduct. Next, solvent evaporation followed by warming in methanol gave the salt (1g) which was dissolved in 25%  $\text{H}_2\text{SO}_4$  and refluxed for 5 hours. Neutralization of the reaction mixture precipitated noroxymorphone (1e) when the reaction sequence was performed without isolation of intermediates, crude noroxymorphone<sup>(18)</sup> was obtained in 98% overall yield from oxymorphone. Allylation of this material with allyl bromide in ethanol gave naloxone in 71% recrystallized yield (70% from 1a). Treatment with ethanolic hydrochloric acid gave naloxone hydro-

Scheme III



Noroxymorphone



Naloxone Hydrochloride

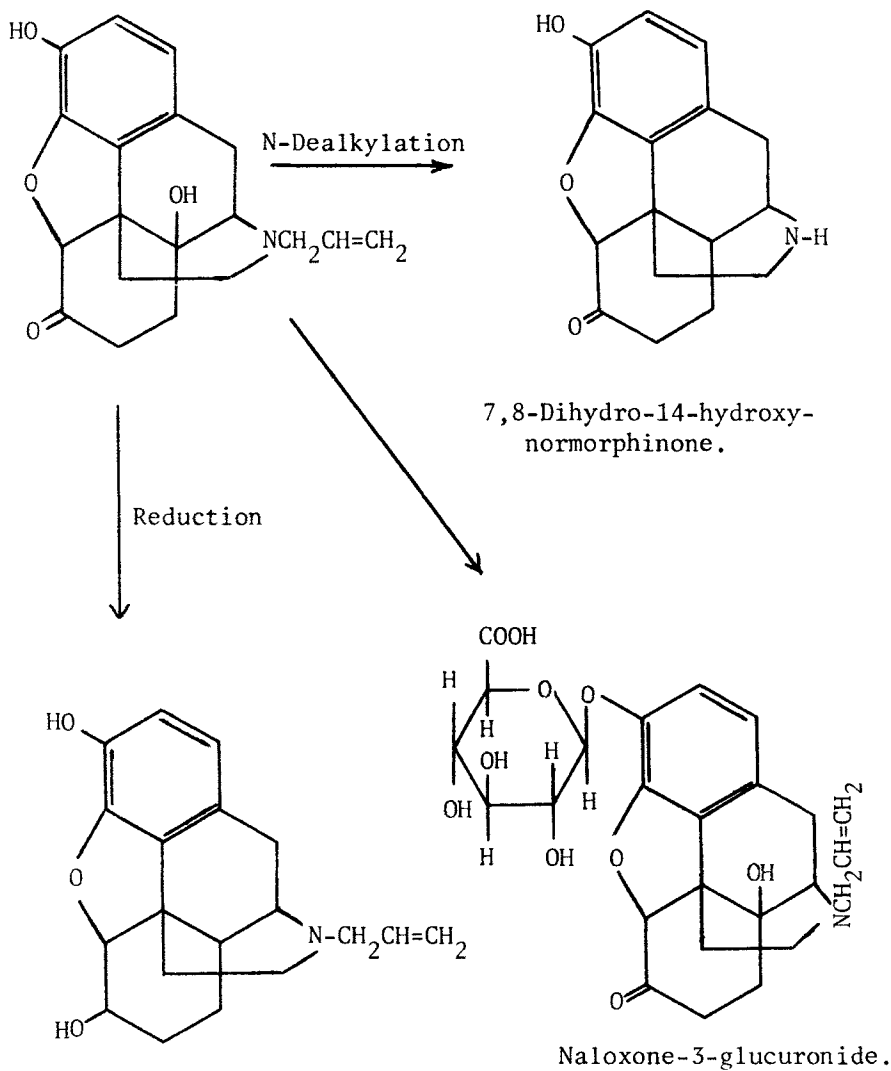
chloride. It should be noted that the isolation of noroxymorphone is achieved by lowering the pH of an aqueous solution to 9 (18) at which point the compound precipitates completely. This very polar substance darkens but does not melt below 300°C and is essentially insoluble in common organic solvents. It has never been successfully recrystallized presumably because of the incompatibility of the ketone and secondary amine functions. No useful purification procedure is available and no simple assay of sample purity exists. For synthetic purposes, this intractable material ordinarily is assumed to be reasonably pure. The difficulty of imagining an equally insoluble reaction by-product or side product provides the main justification for this assumption. Thus, the 98% yield reported here should be viewed with some skepticism. However, it should be noted that the material obtained here is at least as pure as that isolated by other methods. For example, the best previous yield for the allylation step, noroxymorphone to naloxone was 72%. (19)

#### 4. Disposition of Naloxone Hydrochloride

Fishman *et al.* (20) have studied the disposition of naloxone-7,8-<sup>3</sup>H in normal and narcotic dependent men. Naloxone-7,8-<sup>3</sup>H was administered intravenously and orally on separate occasions to the same normal male subject and its disposition was examined. The fate of intravenous naloxone-7,8-<sup>3</sup>H was also studied in an opiate dependent subject both while on heroin maintenance and after withdrawal. In all cases the urinary excretion was rapid but incomplete, never exceeding 70% of the dose over 72 hours. Initial plasma concentration of naloxone were low with a rapid rate of disappearance. Oral naloxone entered plasma quickly but in a metabolised form. The volume of distribution, plasma half-life and metabolic clearance rate of naloxone as calculated from the intravenous studies were about 200 litres, 90 minutes and 2500 litres/day, respectively.

#### 5. Metabolism

Fujimoto (21) isolation of the N-allyl-7,8-dihydro-14-hydroxynormorphine-3-glucuronide metabolite of naloxone in chicken urine aroused speculations concerning its occurrence as a human metabolite. In rabbit and in man, naloxone glucuronide (22) has been reported as the sole metabolite of naloxone. An isolation procedure developed by Fujimoto and his coworkers (23) have been used by



N-Allyl-7,8-dihydro-14-hydroxynormorphine.

## Metabolism of Naloxone

Weinstein et al (24) to separate the metabolites of naloxone. These are identified as the glucuronide of naloxone, 7,8-dihydro-14-hydroxynormorphinone (EN-3169), and N-allyl-7,8-dihydro-14-hydroxynormorphine (EN-2265). The metabolism of phenolic opiates by rat intestine was also reported (25). Results indicated that naloxone has not been dealkylated by rat intestine. An improved method for extraction of naloxone from biological samples was described by Sprague and Takemori (26).

## 6. Methods of Analysis

### 6.1 Identification

- a) Dissolve about 150 mg in 25 ml of water in a small separator, add a few drops of ammonia TS, extract with three 5 ml portions of chloroform, and filter the extracts through a dry filter, collecting the filtrate on a steam bath to dryness, and dry at 105° for 1 hour. The infra-red absorption spectrum of 1 in 50 solution in chloroform of the residue so obtained, determined in a 0.5 mm cell, exhibits maxima only at the same wavelengths as that of a similar solution of Naloxone Reference Standard.
- b) To 1 ml of (1 in 100) solution of naloxone hydrochloride add 1 drop of ferric chloride TS. A clear purplish blue colour is observed.

### 6.2 Titrimetric Methods

#### 6.2.1 Aqueous

A biphasic differential potentiometric titration of naloxone hydrochloride (diprotic acid  $\text{-H}_2\text{A}^+$ ) in presence of molindone hydrochloride (monoprotic acid  $\text{-BH}^+$ ) and m-nitrophenol (lipophilic acid HA) has been described (27) as follows:

A mixture of the test solution containing 0.2 to 0.3 millimole of naloxone hydrochloride in 60 ml of 0.108 M sodium chloride aqueous solution, was deaerated with nitrogen in a titration vessel. Twenty millilitres of pure chloroform was added and the mixture was vigorously stirred during titration with 0.4 M-NaOH; the pH was measured within 10 to 15

seconds after each addition of titrant. A sharp and large pH break was observed at the first equivalence point for naloxone hydrochloride. The equivalence point was evaluated from first derivative plot.

#### 6.2.2 Non-aqueous

USP XIX 1975 (28) described the following procedure:

Dissolve about 300 mg of naloxone hydrochloride accurately weighed, in a mixture of 40 ml of glacial acetic acid and 10 ml acetic anhydride. Add 10 ml of mercuric acetate TS and 1 drop of methyl violet TS, and titrate with 0.1 N perchloric acid VS. Perform a blank determination and make any necessary correction. Each ml of 0.1 N perchloric acid is equivalent to 36.38 mg of naloxone hydrochloride.

### 6.3 Chromatographic Analysis

#### 6.3.1 Combined Thin Layer Chromatography (TLC) and Gas-Liquid-Chromatography (GLC)

DiGregorio and O'Brien (29) developed a chromatographic procedure for the detection of narcotic agonists in human urine. Samples of urine (adjusted to pH 5 to 6) were treated with Amberlite XAD-2 resin or Analtech SA-2 ion exchange paper and subjected to TLC on silica using ethylacetate-methanol-conc. aq.  $\text{NH}_3$  (17:12:1) as a developing solvent. Alternatively the sample of urine was adjusted to pH 10 with 0.1 M - NaOH and extracted with  $\text{CHCl}_3$ . The extract was evaporated to dryness at 60°C. The residue, redissolved in 100  $\mu\text{l}$   $\text{CHCl}_3$ , was subjected to g.l.c. on a column packed with 3% of OV-17 on chromosorb WHP (80 to 100 mesh). The operation was performed at 270°C with nitrogen as a carrier gas at a flow rate of 40 ml  $\text{min}^{-1}$  and a flame ionization detector. The limit of detection attained with both methods was 1  $\mu\text{gml}^{-1}$  for

each drug and the recoveries (from urine) of naloxone, cyclazocine and naltrexone in the range 2.5 to 10  $\mu\text{gml}^{-1}$  were from 80 to 100%.

Cone described a general procedure (30) for the isolation and identification of  $6\alpha$ - and  $6\beta$ -hydroxy metabolites of narcotic agonists and antagonists with a hydromorphone structure. Samples of urine were hydrolysed with 10% conc. HCl at  $115^{\circ}\text{C}$  and 15 p.s.i. for 20 min, and the hydroxymetabolites of naloxone along with oxymorphone, oxycodone, hydromorphone and hydrocodone were isolated by extraction with  $\text{CHCl}_3$ . The separation was carried out by TLC using Celman ITLC types SG or SA plates and a developing solvent composed of  $\text{CHCl}_3$  saturated with aq.  $\text{NH}_3$ . Test strips of the chromatogram were sprayed with potassium iodoplatinate reagent for location of the metabolites. The methanolic extract of the metabolites was evaporated to dryness, redissolved in 0.1 ml of methanol and then analysed by g.l.c. either directly or after silylation. The chromatograph used possessed two columns (6 ft X 2 mm) one packed with 3% of OV-17 and the other with 3% of OV-225 on Gas-Chrom Q. The operation was carried out at  $250^{\circ}\text{C}$  with nitrogen as a carrier gas (30  $\text{ml min}^{-1}$ ) and a flame ionization detector.

### 6.3.2 Gas-Liquid-Chromatography

Naloxone and naltrexone in quantities of 2 to 100 mg have been determined by Sams and Malspeis (31) by g.l.c. using  $^{63}\text{Ni}$  electron capture detector. The procedure involved transference of the methanolic solutions of the drugs to culture tubes; followed by evaporation to dryness under a stream of nitrogen. Twenty-five microlitres of derivatizing agent consisting of heptafluorobutyric, heptafluoropropionic acids or trifluoroacetic anhydride together with 50  $\mu\text{l}$  of 1% pyridine solution in benzene were added. Each tube was heated at  $70^{\circ}\text{C}$  for 2 hrs. and 5 ml of saturated borate buffer was added. The mixture was shaken for 3 min, centrifuged for 4 min and 10  $\mu\text{l}$  of the organic phase was



immediately subjected to g.l.c. on a glass column (183 cm X 2 mm) packed with 3% of OV-1 or OV-17 on Gas-Chrom. (100-120 mesh). A mixture of argon : methane (19:1) was employed as a carrier gas at a flow rate of 40 ml min<sup>-1</sup>. The OV-1 column was temp. programmed from 109° C to 270° C at 4° C min<sup>-1</sup> and the OV-17 column was operated isothermally at 205° C. By the formation of the triester derivative with the heptafluorobutyric or pentafluoropropionic anhydrides; it was possible to determine 10 mg of naloxone with a coefficient of variation of 8.5% and a limit of detection of 2 ng. The nature of the ester formed was confirmed by GC-MS and IR-analysis.

Determination of naloxone in blood plasma alone or in presence of cocaine, codeine and similar narcotic drugs was performed by g.l.c. (32). Naloxone was converted to its silyl derivative prior to chromatographing. The lower limit of sensitivity was 6 ng corresponding to 0.13 µg/ml concentration in plasma.

Gas-liquid-chromatographic analysis of naloxone in biological fluids has also been reported by Meffin and Smith (33). The procedure involved extraction of samples of plasma or whole blood with toluene at pH 9 and the use of naltrexone as an internal standard. The organic phase was then extracted with 0.1 M-H<sub>2</sub>SO<sub>4</sub>; and the acid phase was treated with butylammonium hydroxide and -bromopentafluorotoluene in CH<sub>2</sub>Cl<sub>2</sub>. The resulting derivatives were finally analysed by g.l.c. on a column (1.8 m X 3.2 mm) packed with 3% of OV-17 on Gas-Chrom Q (100-120 mesh) and equipped with <sup>63</sup>Ni as electron capture detector. The retention times were 4.5 and 6.75 min for naloxone and naltrexone respectively. The calibration graph for 5 to 200 ng of naloxone was almost rectilinear. The coefficients of variation were < 4.1% (10 determinations) and the sensitivity and reproducibility of the method compared well with those of radio-immunoassay.

- 6.3.3 The United States Pharmacopoeia, Nineteenth Revision (28) described the following method for the assay of naloxone hydrochloride injection.

Buffer: Dissolve 33.5 g of ammonium chloride in about 150 ml of water contained in 250 ml volumetric flask. Add 42 ml of ammonium hydroxide, dilute with water to volume and mix.

Internal Standard Solution: Prepare a solution of papaverine in chloroform having a concentration of about  $1.3 \text{ mg ml}^{-1}$ .

Standard Preparation: Dissolve a suitable quantity of USP Naloxone RS, accurately weighed, in internal standard solution to obtain a solution having a known concentration of about  $1 \text{ mg ml}^{-1}$ .

Assay Preparation: Pipet a volume of naloxone hydrochloride injection, equivalent to about 6 mg of naloxone hydrochloride, into 60 mg separator, and add 10 ml of water. Adjust the solution to a pH between 8.5 and 9 (using short-range pH paper) by adding 2 ml of buffer. Extract with five 10 ml portions of chloroform and filter the chloroform layer through a funnel containing a pledget of chloroform-wetted cotton into 100 ml round-bottom flask. After the final extraction, rinse the funnel with chloroform, and evaporate the chloroform solution in vacuum on a rotary evaporator with the aid of warm water bath to dryness. Dissolve the residue in 5.0 ml of internal standard solution.

Procedure: Inject separately suitable portions of the standard preparation and the assay preparation into a suitable gas chromatograph equipped with a flame ionization detector and having a 1.2 m X 3 mm (I.D.) glass column that contains 3.8 per cent phase G9 on packing SIA. The column, injection port,

and detector temperatures are maintained at about 230° C, 270° C, respectively. The carrier gas is helium, flowing at the rate of 60 ml min<sup>-1</sup>. Typical retention times for naloxone and papaverine are approximately 4 and 6 minutes respectively.

System Suitability: In a suitable chromatogram, the resolution factor, *R*, is not less than 2.5, between the peaks for naloxone and papaverine and five replicate injections of the standard preparation show a coefficient of variation of not more than 1%.

Calculate the quantity, in mg, of naloxone hydrochloride in the volume of injection taken by the formula  $5(1.111C) (R_u/R_s)$ , in which 1.111 is the ratio of the molecular weight of naloxone hydrochloride to that of naloxone, *C* is the concentration, in mg ml<sup>-1</sup>, of UPS Naloxone RS in the standard preparation, and *R<sub>u</sub>* and *R<sub>s</sub>* are the ratios of the peak heights of the naloxone peak from the assay preparation and standard preparation, respectively.

#### 6.3.4 High-Performance-Liquid-Chromatography (h.p.l.c.)

Peterson *et al.* (34) investigated the amperometric responses of naloxone, naltrexone, morphine, codeine, nalorphine, oxymorphone and pentazocine by injecting the methanolic solution of their hydrochlorides on to a column of  $\mu$ -Bondpack C<sub>18</sub>. Good separation of a mixture of naloxone, morphine, oxymorphone, naltrexone and nalorphine was achieved. Rectilinear calibration graph was obtained for naloxone in the range 5 to 100 ng and the detection limit was 1 pg.

Fluorescence and U.V. detection of naloxone, morphine, etorphine, levallorphan, separated by reversed-phase h.p.l.c., has been reported by Glasel and Venn (35). In this report, naloxone hydrochloride and the other opiates in the form of their water-soluble acid salts were separated on a column (15 cm X 4.6 mm) of Supelco LC-18 operated with 65% acetonit-

rile solution in phosphate buffer solution of pH 5.05 and aqueous 80% acetonitrile mixed in various proportions (2:3 to 3:2) as components of the mobile phase. The separation efficiency was found to depend markedly on the (I) value of the mobile phase. U.V. absorption was monitored at 225 nm whereas fluorescence was monitored at 290 nm (excitation) and at 330 nm (emission).

#### Naloxone Hydrochloride Injection

Hanna et al. (36) analysed the injectable dosage form of naloxone hydrochloride by h.p.l.c. The drug and methyl and propyl-hydroxybenzoates (the preservatives in injectable solution) were separated on a column 30 cm X 6.3 mm of  $\mu$ -Bondpack (10  $\mu$ m) with methanol aqueous 0.1% ammonium carbonate (9:11) as mobile phase of flow rate 3 ml min<sup>-1</sup>. The detection was done at 220 nm and papyrverine was employed as internal standard. The detection limit found was 0.16 mg ml<sup>-1</sup>. The method could also be used to establish the stability of the drug in solution.

An h.p.l.c. method has also been developed by Tawakkol et al. (37) for the analysis of injectable solution of naloxone hydrochloride (Narcane-Neonatal Injectable<sup>R</sup>). The column used was C<sub>18</sub>, Micropack (MCH-10 (monomeric) and the mobile phase was acetonitrile : 0.01 M KH<sub>2</sub>PO<sub>4</sub> (70:30) at a flow rate of 2 ml min<sup>-1</sup>. Authentic naloxone hydrochloride was used to establish a calibration curve with linear relationship in the concentration 10 to 50  $\mu$ g/ml<sup>-1</sup>. The retention time for naloxone hydrochloride was 3.3 min. Average percentage recovery obtained was 99.0  $\pm$  2.6.

#### 6.3.5 Radio Immunoassay (RIA)

An RIA was developed (38) and was applicable for naloxone in either serum or brain. The limit of sensitivity of the assay was 0.1 ng. Naloxone glucuronide, nornaloxone and morphine were not recognized by the antibody whereas naltrexone and 6-hydroxynaloxone were

able to displace  $^3\text{H}$ -labelled naloxone from the antibody. The assay was of sufficient sensitivity to follow the serum levels of naloxone in man for upto 2 hrs. after an i.v. injection of 0.4 mg. In animal studies, the biologic half-lives of naloxone or morphine (5 mg/kg) were compared after s.c. injection in rats. The peak serum levels ( $1\text{ }\mu\text{g ml}^{-1}$ ), time to peak serum levels ( $< \frac{1}{2}$  hr.), and serum half-life (40 min) were comparable. However, the brain entry and egress of the two compounds differed markedly. Peak brain levels of naloxone occurred within 15 min. and declined by 50% within 1 hr., whereas the peak brain levels of morphine were sustained for upto 2 hrs. At peak serum levels, the brain serum ratio for morphine was 0.1 whereas for naloxone it was 15 times greater. Thus, the high brain/serum ratio of naloxone contributes to its potency whereas the rapid egress from the brain is important in the short duration of action of naloxone.

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# DIFLUNISAL

Meredith L. Cotton and Robert A. Hux

*Merck Frosst Canada Inc.  
Pointe Claire-Dorval, Quebec, Canada*

1. Introduction	492
1.1 History	492
1.2 Name, Formula, Molecular Weight	492
1.3 Appearance, Color, Odor	492
2. Physical Properties	493
2.1 Crystal Properties	493
2.2 Thermal Behavior	494
2.3 Solubility	495
2.4 Acid Dissociation Constants	497
2.5 Hygroscopicity	497
2.6 Vapor Pressure	497
2.7 Infrared Spectrum	497
2.8 Magnetic Resonance Spectra	503
2.9 Ultraviolet Spectrum	509
2.10 Mass Spectrum	509
3. Synthesis	509
4. Stability and Degradation	509
4.1 Solid-State Stability	509
4.2 Solution Stability	513
5. Pharmacokinetics and Metabolism	513
5.1 Pharmacokinetics	513
5.2 Metabolism and Elimination	514
5.3 Interactions	514
6. Methods of Analysis	515
6.1 Identification Tests	515
6.2 Titrimetric Assay	515
6.3 Ultraviolet Spectrophotometric Analysis	515
6.4 Fluorescence Analysis	515
6.5 Ferric Nitrate Colorimetric Assay	516
6.6 Chromatographic Analysis	516
6.7 Determination in Biological Fluids	523
References	524

## 1. INTRODUCTION

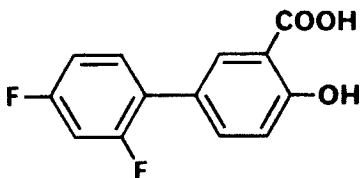
### 1.1 History

Diflunisal, an analgesic anti-inflammatory agent developed by Merck Sharp & Dohme Research Laboratories, is the result of an attempt to identify a salicylic acid derivative superior to aspirin with improved potency, tolerance and duration of action. Although diflunisal, as a salicylate, has many pharmacological attributes similar to those of aspirin, its two primary structural differences from aspirin may account for its different biochemical response. The presence of the difluorophenyl group probably enhances its potency and duration of action, whereas the absence of the O-acetyl group may account for its significantly lower toxicity. Lacking the O-acetyl function, diflunisal is incapable of enzyme acetylation. It influences the prostaglandin synthetase pathway differently and has a reduced effect on platelet function when compared with aspirin (1). Several review articles outline its chemistry and pharmacology (2-7).

### 1.2 Name, Formula, Molecular Weight

The name for diflunisal (MK-647) used in Chemical Abstracts is 2',4'-difluoro-4-hydroxy-[1,1'-biphenyl]-3-carboxylic acid. The CAS registry number is 22494-42-4. Other names include 5-(2',4'-difluorophenyl)salicylic acid, 2',4'-difluoro-4-hydroxy-3-biphenylcarboxylic acid, 2-(hydroxy)-5-(2,4-difluorophenyl)benzoic acid, 'DOLOBID', Dolobis, Flovacid and Fluniget (8).

Its structural formula is:



$C_{13}H_8F_2O_3$

Molecular weight: 250.20

### 1.3 Appearance, Color, Odor

Diflunisal is an odorless, white, crystalline compound melting at 211-213°C. It fluoresces on exposure to short wavelength (254 nm) and long wavelength (366 nm) ultraviolet light.

## 2. PHYSICAL PROPERTIES

### 2.1 Crystal Properties

#### 2.1.1 Crystallinity

Diflunisal is polymorphic. It is known to exist in three nonsolvated crystal forms designated as I, II and III (9). The two most common forms, I and II, are enantiotropic with a transition temperature of 98°C determined from aqueous solubility data at different temperatures. Form I is the stable form above 98°C and Form II is the stable form at ambient conditions (9). Forms II and III also are enantiotropic with a transition temperature estimated from solubility studies to be about 80°C. Because of its inherent instability, Form III is difficult to obtain. It is monotropic with Form I with no apparent transition temperature below the melting point (9). Since Form II is the thermodynamically more stable form at room temperature to which Forms I and III will eventually revert, it is the preferred common crystal form of the drug.

#### 2.1.2 X-Ray Diffraction

The X-ray diffraction patterns of the polymorphic forms were obtained (10) using a Phillips-Norelco diffractometer with copper K $\alpha$  radiation (1.5418 Å). The data for each crystal form are in Tables I, II and III.

#### 2.1.3 Scanning Electron Microscopy

Form II consists of long, thin acicular crystals with rectangular cross sections when viewed using an International Scientific Instrument ISI-40 scanning electron microscope (11).

TABLE I

X-Ray Diffraction Data for Polymorphic Form I of Diflunisal

<u>2<math>\theta</math></u>	<u>2Sine</u>	<u>d(Å)</u>	<u>I/I<math>_0</math> 100</u>
4.1	0.0715	21.5	100
8.2	0.143	10.7	7
12.4	0.216	7.1	21
13.2	0.230	6.7	90
14.3	0.249	6.2	50
14.7	0.256	6.0	21
16.5	0.287	5.4	44
17.0	0.296	5.2	67
19.3	0.335	4.6	4
19.9	0.346	4.5	2
20.7	0.359	4.3	13

TABLE IIX-Ray Diffraction Data for Polymorphic Form II of Diflunisal

<u>2<math>\theta</math></u>	<u>2Sine</u>	<u>d(Å)</u>	<u>I/I<sub>o</sub> 100</u>
4.8	0.0838	18.4	27
7.2	0.126	12.2	7
9.7	0.169	9.1	3
10.3	0.180	8.6	1
11.9	0.207	7.4	32
13.1	0.228	6.8	23
14.6	0.254	6.1	100
16.3	0.284	5.4	29

TABLE IIIX-Ray Diffraction Data for Polymorphic Form III of Diflunisal

<u>2<math>\theta</math></u>	<u>2Sine</u>	<u>d(Å)</u>	<u>I/I<sub>o</sub> 100</u>
7.6	0.133	11.6	12
10.8	0.188	8.2	13
12.5	0.218	7.1	69
13.2	0.230	6.7	2
14.1	0.245	6.3	5
15.3	0.266	5.8	100
16.7	0.290	5.3	17
17.7	0.308	5.0	2
18.2	0.316	4.9	1
19.9	0.346	4.5	8

2.2 Thermal Behavior

Diflunisal has been observed to melt at 211-213°C with sublimation. Using a Perkin-Elmer DSC-4 differential scanning calorimeter, a thermal curve obtained for Form I contains a single endothermic melting transition with an onset temperature of 212°C. The thermal curve for Form II contains a similar endothermic response (Figure 1). This is a result of the rapid conversion of Form II to Form I during heating and the subsequent melting of Form I. The observed shoulder on the endotherm corresponds to the melting of unconverted Form II (12).

Employing a Zeiss D-7082 optical microscope with a Mettler FP-82 hot stage, samples of Form II were heated over the range of temperature 90-220°C (11). No change in behavior was apparent below 150°C. Maintaining isothermal condi-

tions of 190°C, large, lamellar, birefringent crystals (Form I) formed within minutes and coexisted with the long, rectangular, rod-like crystals originally present (Form II). Heating the sample comprising these two crystal forms, the following melting transitions were observed: 209-211°C (Form II) and 212-214°C (Form I) (11).

### 2.3 Solubility

The approximate solubilities determined at room temperature in organic solvents are shown in Table IV (13).

TABLE IV  
SOLUBILITIES IN ORGANIC SOLVENTS

<u>Solvent</u>	<u>Approximate Solubility</u> <u>(mg/mL)</u>
methanol	>100
ethanol	>100
ethyl acetate	> 50
acetone	> 50
propylene glycol	> 25
chloroform	5
carbon tetrachloride	5
methylene chloride	1.6
hexane	<0.1

TABLE V  
SOLUBILITIES IN AQUEOUS BUFFERS

<u>Buffer</u>	<u>pH</u>	<u>Solubility</u> <u>(mg/mL)</u>
0.1M phosphate	7.05	3.43
0.1M phosphate	6.73	1.51
0.1M citrate	5.77	0.49
0.1M acetate	5.60	1.48
0.1M phosphate	5.48	0.35
0.1M acetate	4.96	0.41
0.1M phosphate	4.85	0.14
0.1M citrate	4.82	0.16
0.1M hydrochloric acid	1.0	0.0083

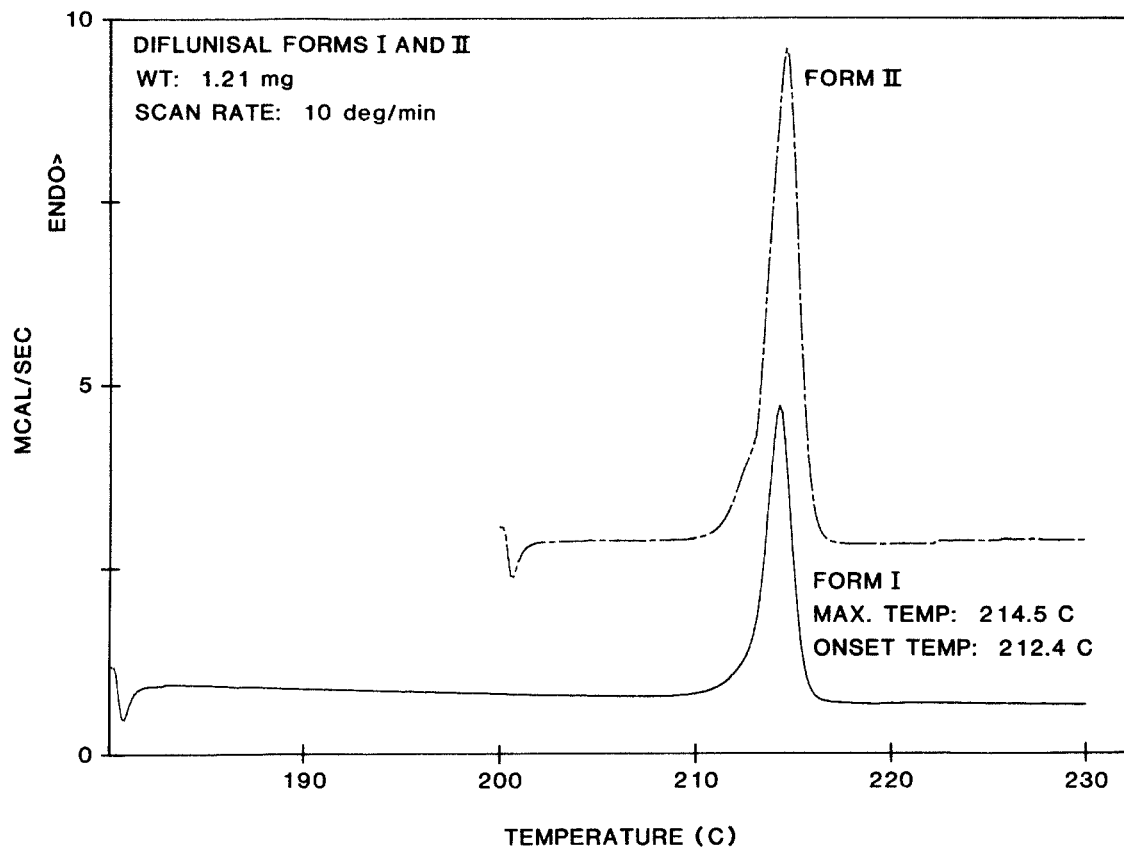


Figure 1. Thermal Curves of Form I and Form II of Diflunisal.

Aqueous solubilities obtained as a function of pH at room temperature in buffered solutions are shown in Table V (13). For unknown reasons, results obtained in acetate buffers were significantly higher than those obtained in citrate buffers.

The solubilities of Forms I, II and III were determined as a function of temperature (see Figures 2, 3). From these solubility data the transition temperatures of Form I and Form II (98°C) and Form II and Form III (80°C) were estimated (9).

#### 2.4 Acid Dissociation Constants

From the solubility data in acetate buffers and the limiting solubility of the free acid in hydrochloric acid solution, a pKa value of 3.3 was estimated for solutions of 0.1 ionic strength (13). The pKa of the phenolic group has been estimated by a spectrophotometric method to be 14. These values correlate reasonably well with pKa values for salicylic acid of 3.0 and 13.9.

#### 2.5 Hygroscopicity

Diflunisal is nonhygroscopic (13). No significant weight gain was observed when samples were stored for 8 days at room temperature and relative humidities of 11%, 33%, 47% and 76%.

#### 2.6 Vapor Pressure

The sublimation pressure of diflunisal was determined using the Knudsen effusion technique and a DuPont thermobalance in the range of temperature 120-195°C and at a pressure of <0.001 mm of Hg (9). The sublimation pressures of Form I and Form II were similar. This was most likely due to the conversion of Form II to Form I under experimental conditions. By extrapolation, the sublimation pressures at 30°C and 40°C were  $1.6 \times 10^{-7}$  mm of Hg and  $7.5 \times 10^{-7}$  mm of Hg, respectively.

#### 2.7 Infrared Spectrum

Infrared spectra of the three polymorphic forms are shown in Figures 4-6. The spectra were obtained using a Perkin-Elmer 521 spectrophotometer (10). The characteristic bands of each spectrum with assignments are listed in Table VI.

The infrared spectrum can be used to determine the content of Form II in the bulk chemical. Figure 7 shows the relationship between the ratio of the peak heights at 1210



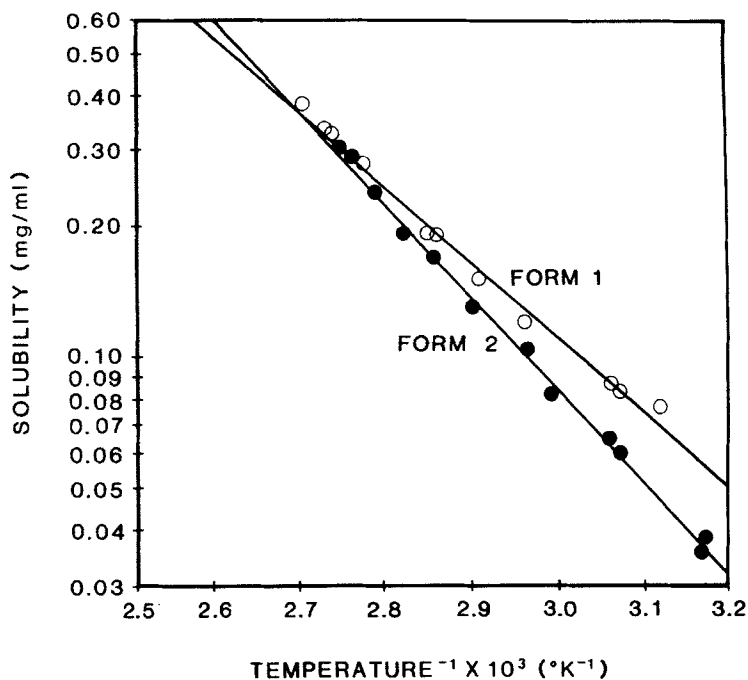


Figure 2. The Aqueous Solubility of Diflunisal, Forms I and II as a Function of Temperature.

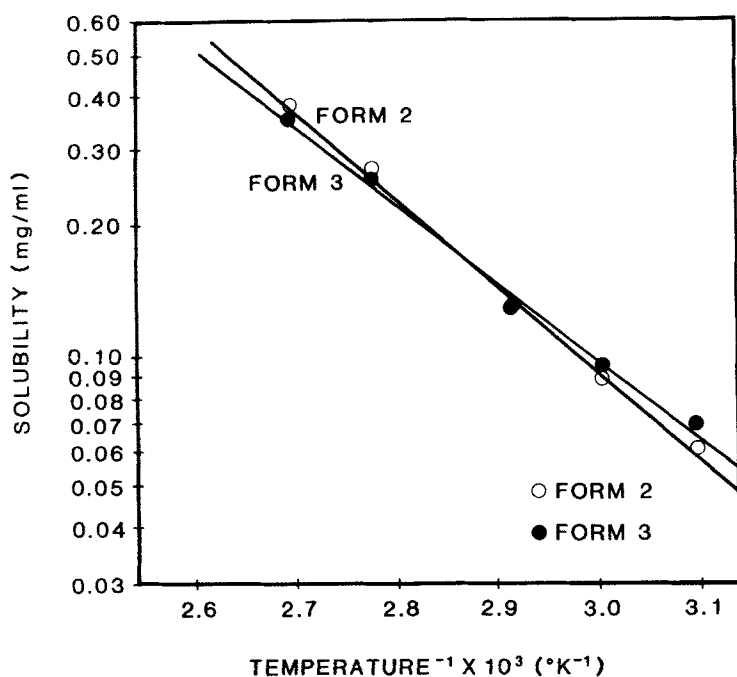


Figure 3. The Aqueous Solubility of Diflunisal, Forms II and III as a Function of Temperature.

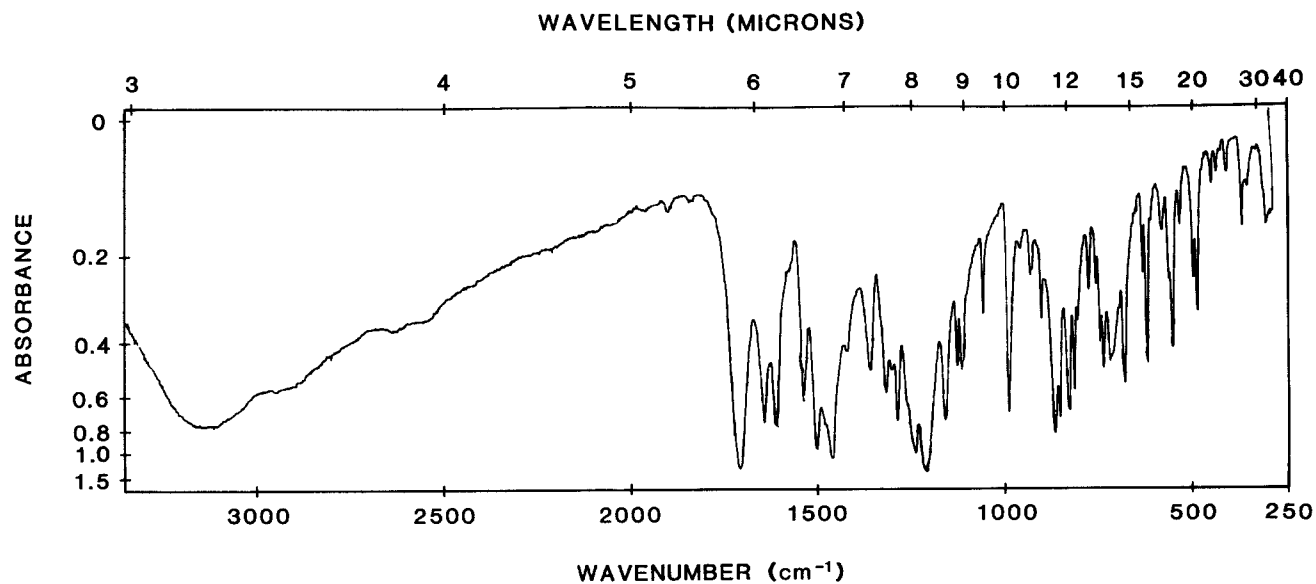


Figure 4. Infrared Absorption Spectrum of Diflunisal, Form I.

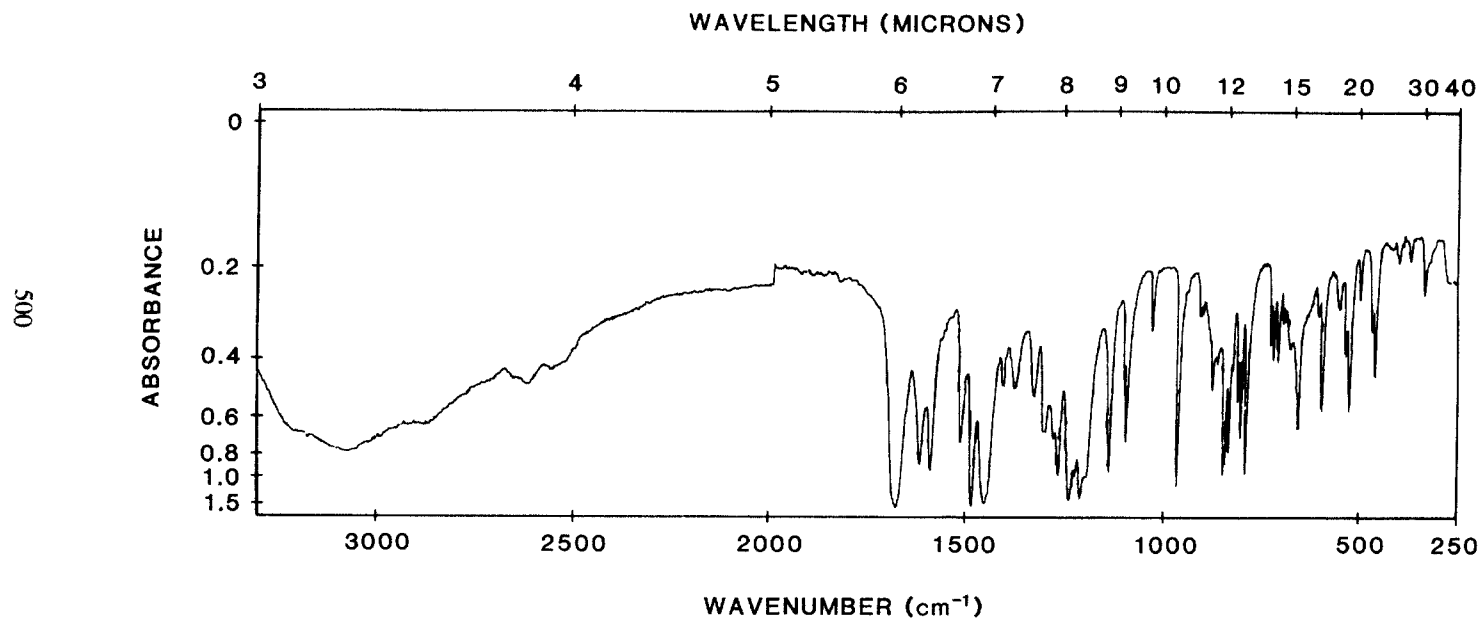


Figure 5. Infrared Absorption Spectrum of Diflunisal, Form II.

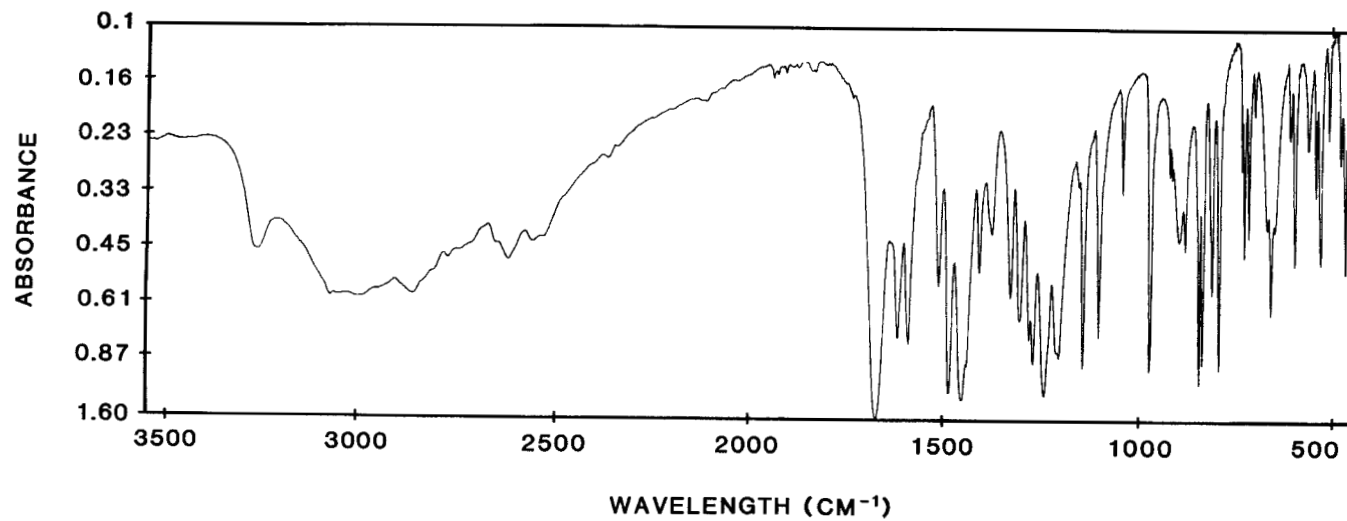


Figure 6. Infrared Absorption Spectrum of Diflunisal, Form III.

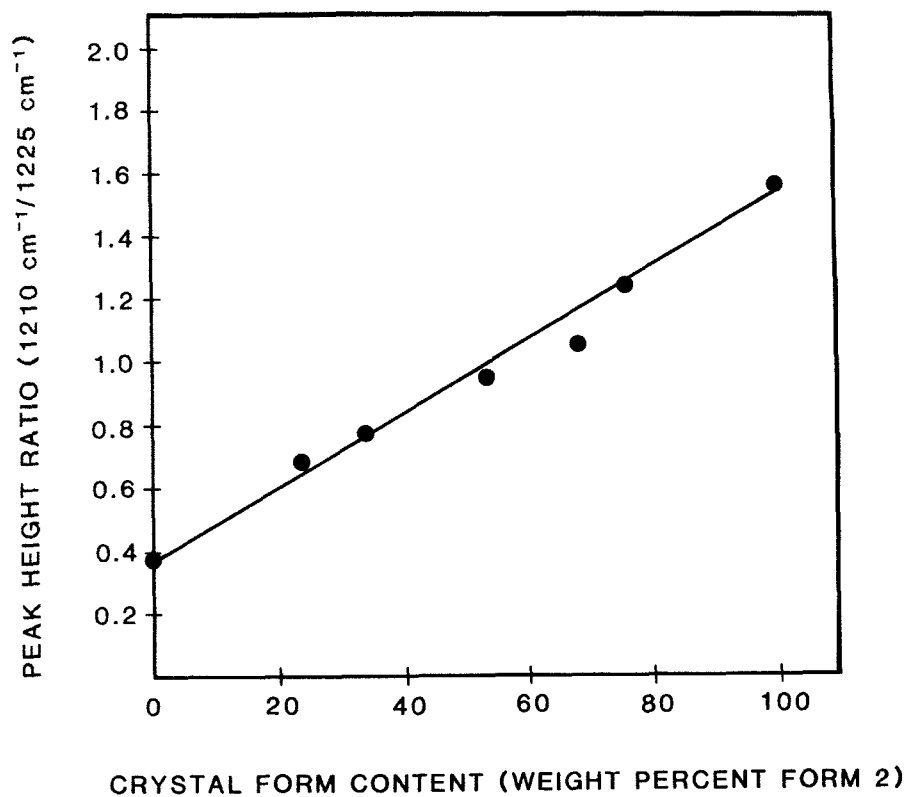


Figure 7. The Determination of Crystal Form Content (Forms I and II) of Diflunisal by Infrared Absorption at 1210 cm<sup>-1</sup> and 1225 cm<sup>-1</sup>.

$\text{cm}^{-1}$  and  $1225 \text{ cm}^{-1}$  and the weight percent of Form II present (14).

TABLE VI

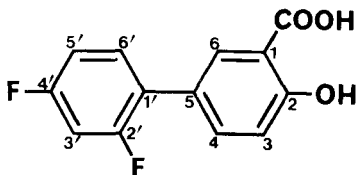
Infrared Spectral Assignments for Diflunisal

<u>Frequency (<math>\text{cm}^{-1}</math>)</u>	<u>Assignment</u>
2800-3200	O-H association and stretching; aromatic C-H stretch
1670	carbonyl stretch
1600	stretch in the phenyl nucleus
1220	C-F stretch
800-850	C-H out-of-plane wag

2.8 Magnetic Resonance Spectra2.8.1 Proton Spectrum

A 70 mg/mL diflunisal solution in  $\text{DMSO}-d_6$  was used to obtain a proton magnetic spectrum (Figures 8, 9) employing a Bruker WM-250 spectrometer with a frequency of 250.13 MHz and tetramethylsilane reference. The general characteristics of the spectrum with assignments are summarized in Table VII (15).

TABLE VII

Proton Magnetic Resonance Assignments for Diflunisal

<u>Chemical Shift, <math>\delta</math> (ppm)</u>	<u>Relative No of Protons</u>	<u>Assignment</u>
7.95 (dd)	1	H <sub>6</sub>
7.68 (ddd)	1	H <sub>4</sub>
7.59 (td)	1	H <sub>6'</sub>
7.34 (ddd)	1	H <sub>3'</sub>
7.18 (tdd)	1	H <sub>5'</sub>
7.09 (d)	1	H <sub>3</sub>
11.2 (s)	2	COOH, ArOH

2.8.2 Carbon-13 Spectrum

The carbon-13 spectrum (Figures 10, 11) was ob-

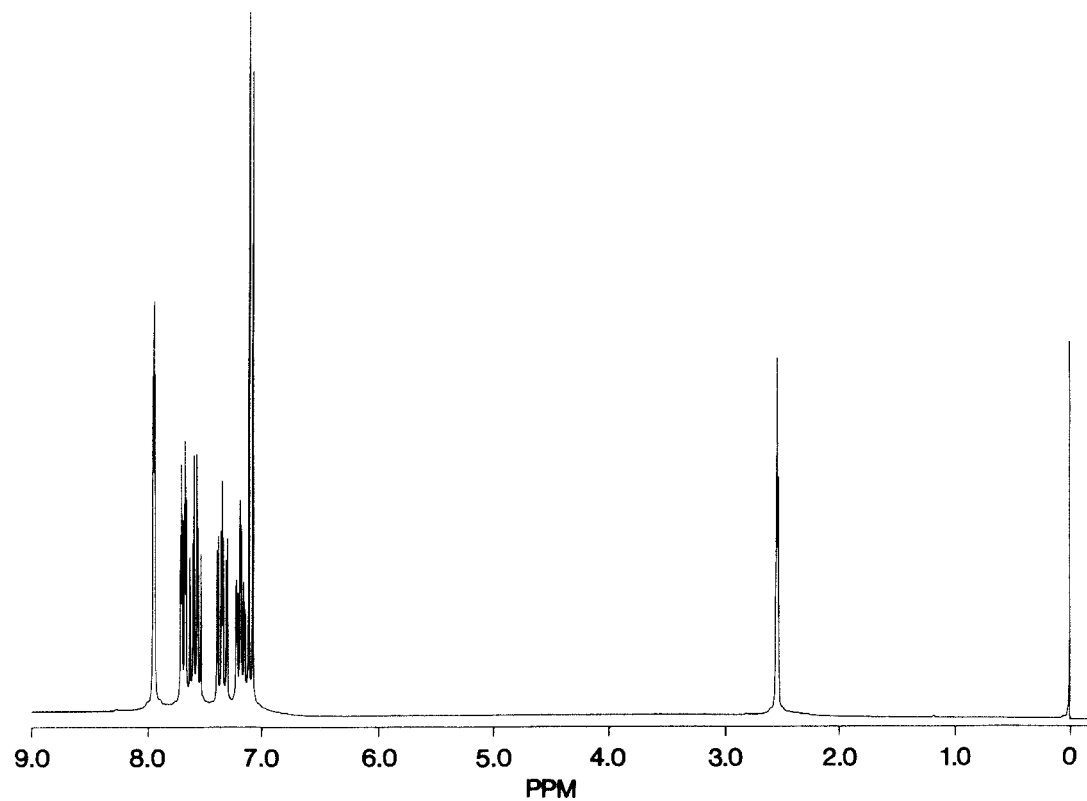


Figure 8. The Proton Magnetic Resonance Spectrum of Diflunisal.

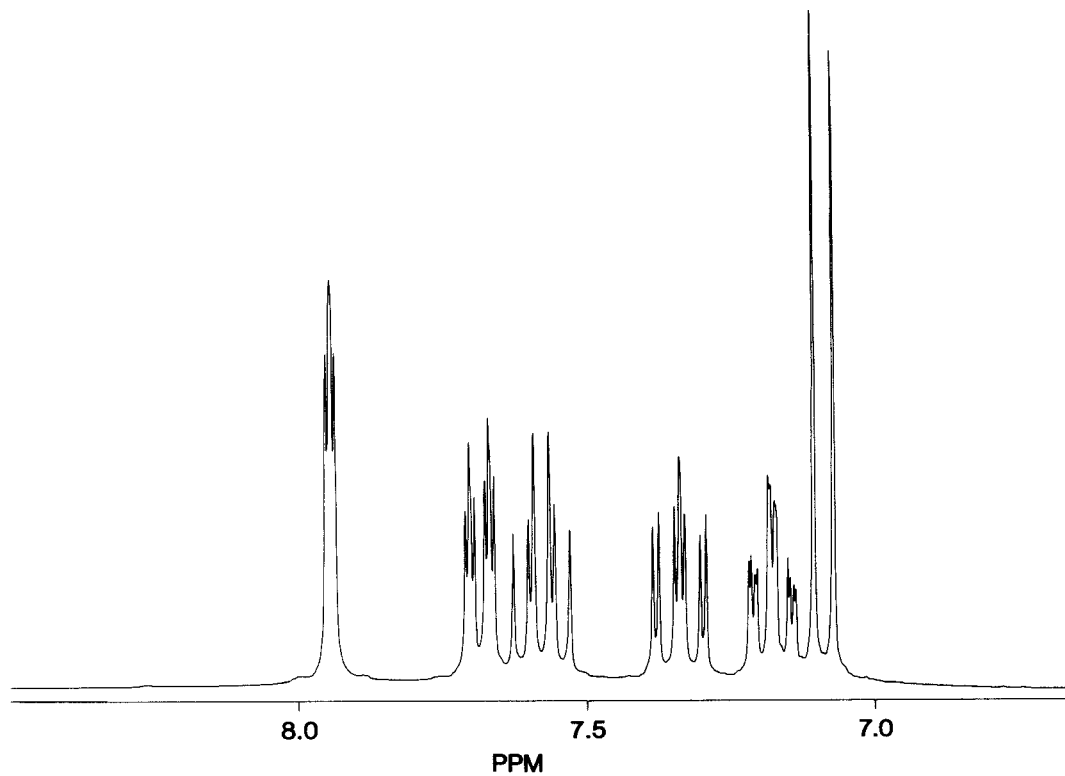


Figure 9. The Proton Magnetic Resonance Spectrum of Diflunisal with Scale Expansion in the Region of 7.0-8.0 PPM.



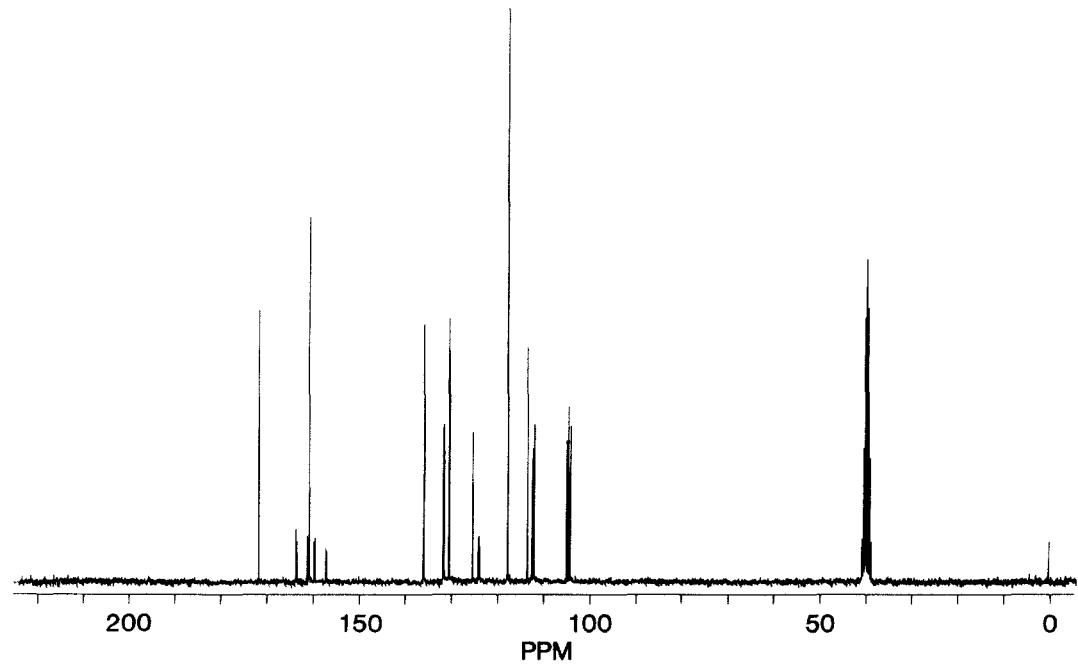


Figure 10. The Carbon-13 Magnetic Resonance Spectrum of Diflunisal.

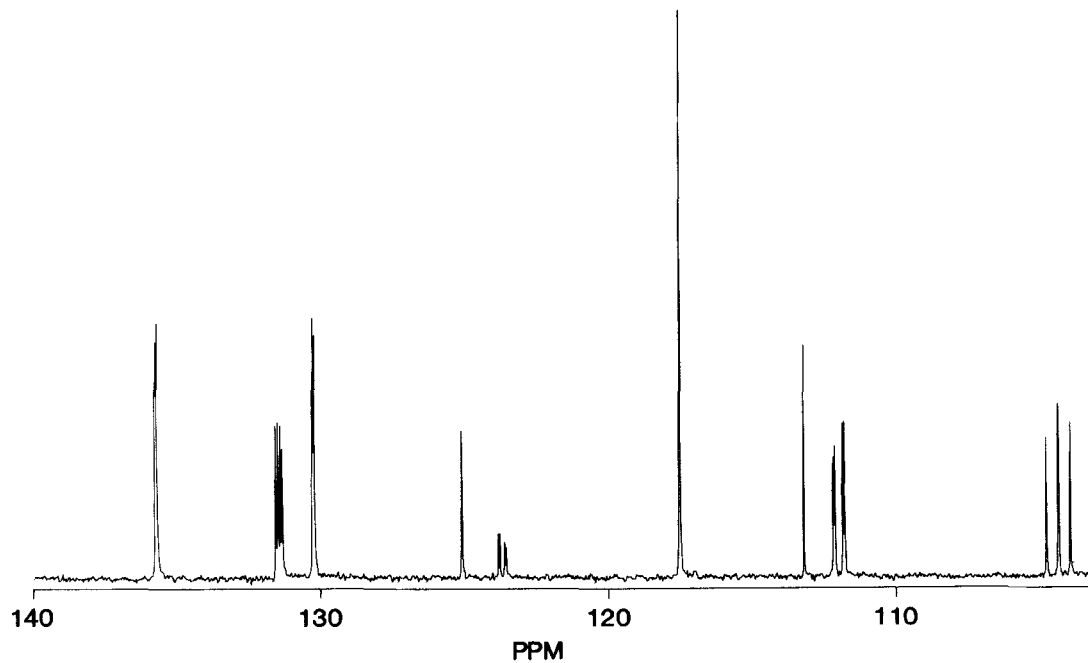
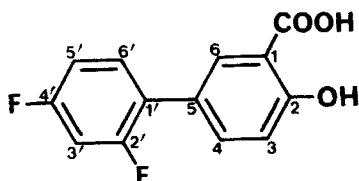


Figure 11. The Carbon-13 Magnetic Resonance Spectrum of Diflunisal with Scale Expansion in the Region of 100-140 PPM.

tained using the same conditions as those indicated for the proton spectrum. The spectral assignments are listed in Table VIII (15).

TABLE VIII

Carbon-13 Magnetic Resonance Assignments for Diflunisal



<u>Carbon</u>	<u>Chemical Shift, <math>\delta</math> (ppm)</u>	<u>C13-F19 Splitting Pattern</u>
COOH	171.5	-
C <sub>1</sub>	113.4	-
C <sub>2</sub>	160.7	-
C <sub>3</sub>	117.5	-
C <sub>4</sub>	135.7	2.7 (F <sub>2'</sub> )
C <sub>5</sub>	125.1	-
C <sub>6</sub>	130.2	3.2 (F <sub>2'</sub> )
C <sub>1'</sub>	123.7	13.8 (F <sub>2'</sub> ), 3.9 (F <sub>4'</sub> )
C <sub>2'</sub>	159.1	248 (F <sub>2'</sub> ), 12.7 (F <sub>4'</sub> )
C <sub>3'</sub>	104.4	26.5 (F <sub>2'</sub> ), 26.5 (F <sub>4'</sub> )
C <sub>4'</sub>	161.4	11.8 (F <sub>2'</sub> ), 247 (F <sub>4'</sub> )
C <sub>5'</sub>	112.0	3.3 (F <sub>2'</sub> ), 20.9 (F <sub>4'</sub> )
C <sub>6'</sub>	131.4	4.9 (F <sub>2'</sub> ), 9.8 (F <sub>4'</sub> )

2.8.3 Fluorine-19 Spectrum

The fluorine-19 spectrum was obtained using a JEOL (USA) C-60HL spectrometer operating at 56.45 MHz with C<sub>6</sub>F<sub>6</sub> internal reference,  $\phi$  = 162.6 ppm. A 1.0 molar solution of diflunisal in DMSO d<sub>6</sub> was used.

The spectrum consisted of two multiplets centered at  $\phi$  = 111.5 ppm and  $\phi$  = 114.1 ppm calculated relative to CFC1<sub>3</sub>. The multiplet at  $\phi$  = 111.5 ppm was assigned to the para-fluorine split by three protons and the other fluorine in the aromatic ring. The pattern centered at  $\phi$  = 114.1 was assigned to the ortho-fluorine split by three protons and the para-fluorine. This pattern was split further by protons in the 4 and 6 positions of the adjacent aromatic ring giving a fine structure pattern (15).

## 2.9 Ultraviolet Spectrum

The ultraviolet absorption spectrum of the protonated acid form was obtained using a Beckman UV 52 spectrophotometer in N/10 hydrochloric acid with methanol as the solvent. This spectrum (Figure 12) is characterized by three well defined maxima at 315 nm, 251 nm and 227 nm with A (1%, 1cm) values of 130, 560 and 1050, respectively (13, 16). The spectrum of the unprotonated conjugate base obtained in N/10 aqueous sodium hydroxide is characterized by two maxima at 273 nm and 221 nm with A (1%, 1cm) values of 455 and 673, respectively (16).

## 2.10 Mass Spectrum

The mass spectrum of diflunisal was obtained using a mass spectrometer in the electron impact mode with 70eV ionizing energy and a probe temperature of 150°C. The output from the mass spectrometer was analyzed and its primary features presented as a bar graph in Figure 13 (17).

The spectrum shows an abundant molecular ion at m/e 250. The base peak at m/e 232 results from a loss of water typical of enolized keto acids. Characteristic peaks at m/e 204 and m/e 175/176 indicate consecutive loss of CO and HCO from m/e 232. A proposed fragmentation pattern is shown in Figure 14 (17).

## 3. SYNTHESIS

Diflunisal is prepared in a sequence of reactions beginning with the synthesis of 2,4-difluorobiphenyl from 2,4-fluoroaniline and isopropyl nitrite (18). The acetyl derivative is then prepared, oxidized and the resulting ester saponified. Carboxylation of the 2,4-difluoro-4-hydroxy-[1,1'-diphenyl] derivative to yield the 3-carboxylic acid completes the synthetic preparation. Several similar variations on this reaction sequence (Figure 15) are described elsewhere (6, 18, 19).

## 4. STABILITY AND DEGRADATION

### 4.1 Solid-State Stability

Diflunisal is a very stable compound in the solid-state. Under ambient conditions of temperature and humidity diflunisal is stable for several years with no indication of degradation by HPLC analysis. Attempts to degrade the compound at 60°C for 7 days or at 105°C for 24 hours did not generate detectable degradation products (13). Neither was degradation observed when the solid was exposed for one week to high intensity fluorescent light (13).

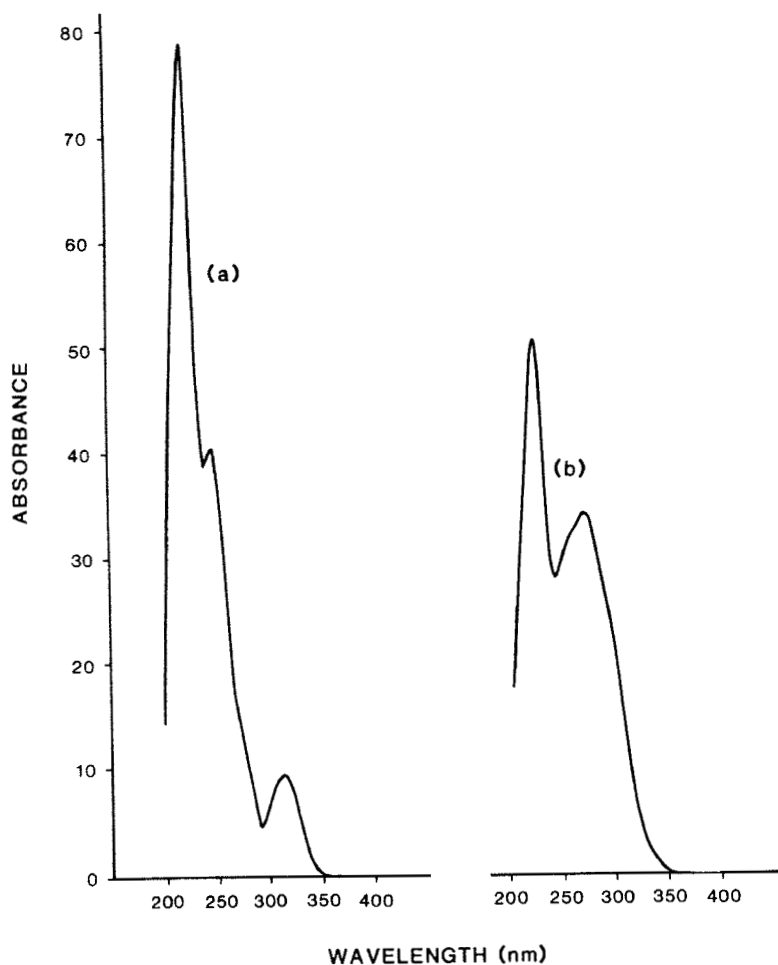


Figure 12. The Ultraviolet Absorption Spectrum of Diflunisal in (a) N/10 Methanolic Hydrochloric Acid; Concentration: 0.764 mg 100 mL and (b) N/10 Sodium Hydroxide; Concentration: 0.754 mg/100 mL.

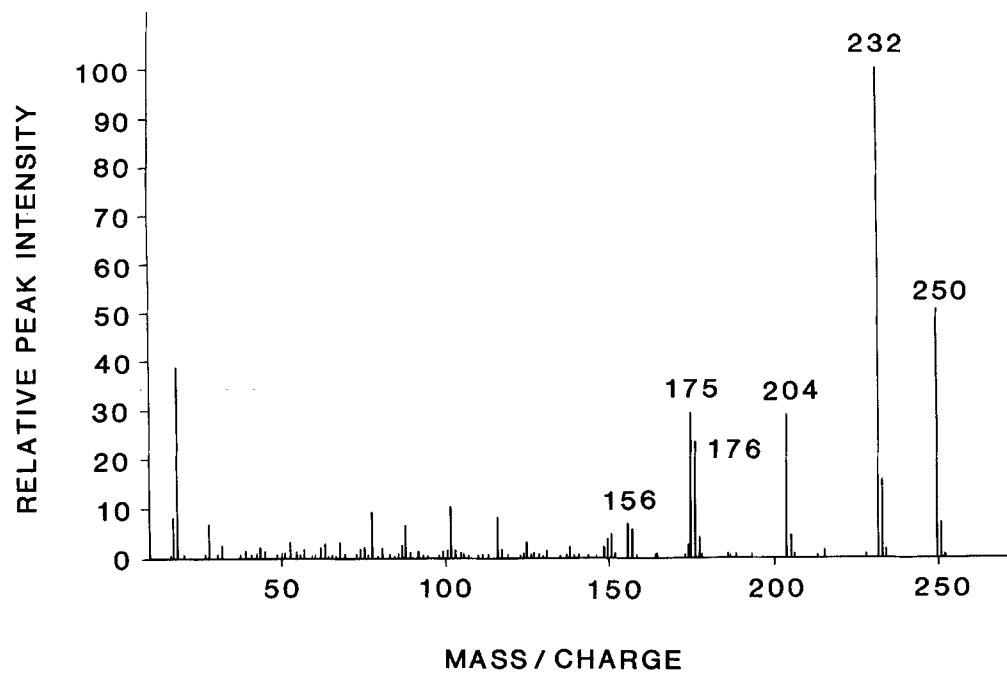


Figure 13. The Low Resolution Mass Spectrum of Diflunisal.

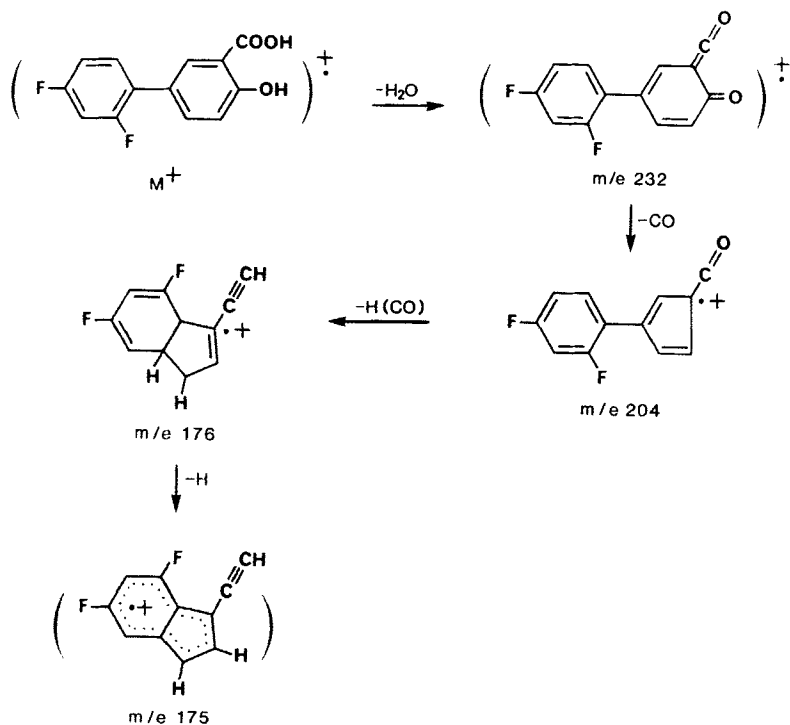


Figure 14. A Proposed Fragmentation Pattern to Explain the Mass Spectrum of Diflunisal.

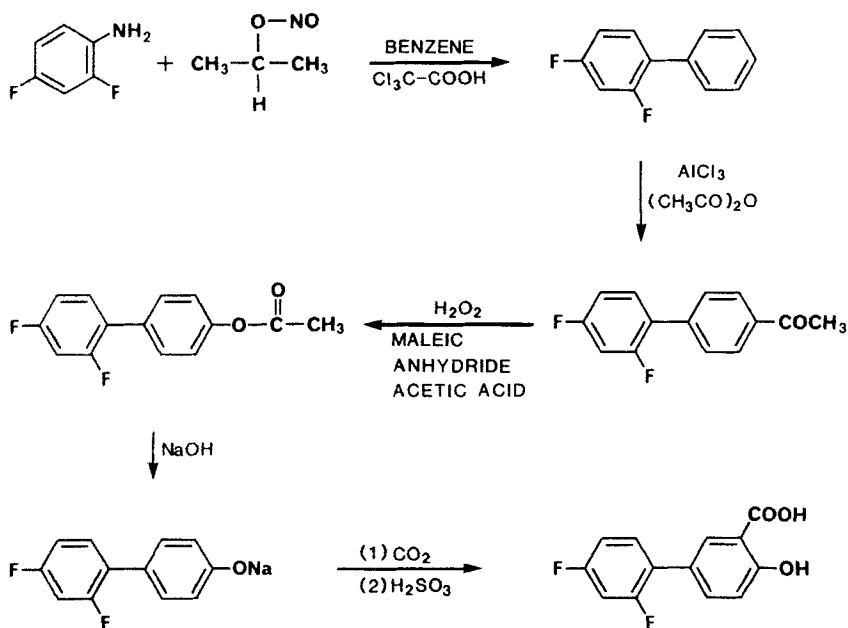


Figure 15. Synthesis of Diflunisal.

Decarboxylation can occur at conditions of severe thermal stress and appears to be the primary thermal degradation process. Heating diflunisal in sealed glass ampoules at 230°C for 20 minutes produced two minor degradation products. One degradation product resulting from decarboxylation was 4(2',4'-difluorophenyl)phenol or descarboxydiflunisal. The second compound, the diflunisal descarboxydiflunisal ester, was formed from the first product reacting with diflunisal. Analysis for intact diflunisal in this severely stressed sample using HPLC in one case and a colorimetric method with ferric ion complexation in another case, indicated less than one percent degradation (20).

Exposure of the sodium salt of diflunisal in polyethylene glycol suppository base to intense ultraviolet light for 16 hours produced minor amounts of the sodium salt of the desphenol, 3(2',4'-difluorophenyl)benzoic acid (21).

#### 4.2 Solution Stability

Acidic methanol solutions result in a slow rate of ester formation. However, solutions of diflunisal in methanol and aqueous methanol are stable for several days at ambient conditions. A solution in methanol exposed to normal room lighting for 9 months showed a slight increase in solution color.

Oxidation of the phenolic group of diflunisal is a possible degradative pathway in solution. Aqueous ethanol solutions containing one percent potassium permanganate after 24 hours showed one additional spot formed from polar material remaining at the origin of a TLC plate. Analysis by UV spectrophotometry and a ferric nitrate colorimetric method indicated 78% of the initial diflunisal remained intact (21).

### 5. PHARMACOKINETICS AND METABOLISM

#### 5.1 Pharmacokinetics

##### 5.1.1 Absorption

Diflunisal after oral administration in doses of 50-500 mg was well absorbed (3, 22, 23). Peak plasma levels of 85-100 µg/mL were obtained about two hours after administration (2, 23-26). The oral bioavailability was essentially 100% based on urinary recovery after 96 hours (23). Diflunisal administered orally with food showed a 20 minute time delay and a 16% reduction in peak plasma concentrations but the AUC remained unchanged (27). The time required to reach the steady-state was dose-dependent,



increasing from 3-4 days with a 125 mg twice daily dose to 7-9 days with a 500 mg twice daily dose (23). Minimum plasma steady-state concentrations increase from 13  $\mu\text{g/mL}$  for 125 mg twice daily dose to 100  $\mu\text{g/mL}$  for a 500 mg twice daily dose (28).

#### 5.1.2 Distribution

Although there is limited information on the distribution of diflunisal in man, it has been shown to be highly bound to plasma proteins (23) in normal subjects (>99%) (29). The apparent volume of distribution was low (0.1 liter/kg) (30) and, in lactating women, its concentration in milk was 2-7% of its concentration in plasma (2).

#### 5.2 Metabolism and Elimination

Elimination is concentration-dependent and very much dependent upon conjugation with glucuronic acid. About 80-95% of an oral dose is excreted in the urine 72-96 hours following administration, primarily as the phenolic and acyl glucuronides (23). The principal metabolite is the phenolic glucuronide conjugate of diflunisal accounting for 64% of the radioactivity in the urine after doses of 50-500 mg of radiolabelled diflunisal (23). The ester glucuronide conjugate accounts for 20% of urinary radioactivity (23). Total body clearance is small (7.9 mL/min) (30). Less than 5% of a single dose is recovered in the feces (3) from either unabsorbed drug or biliary excretion.

Elimination is dose-dependent. The terminal half-life in plasma ranges from 5 hours for a 50 mg oral dose to 15 hours for a one gram dose (23). The half-life increases considerably with renal impairment (26, 31, 32).

Diflunisal is not metabolized to salicylic acid by cleavage of the two aromatic centers nor is there any evidence for loss of fluorine (23). No evidence exists for the glycine conjugate of diflunisal although it is a major route of elimination in salicylate metabolism.

#### 5.3 Interactions

The bioavailability of diflunisal is significantly decreased by aluminum hydroxide gel in fasted subjects but this antacid effect is significantly less in patients who have eaten (33-35). Concomitant administration of aspirin with diflunisal decreases plasma concentrations of diflunisal but this effect may not be of clinical importance (2, 36). Because diflunisal is highly bound to plasma

protein, it has the potential for interacting with other protein-bound agents. A 500 mg twice daily dose for 2 weeks increases the percentage of warfarin unbound to protein and lowers total plasma warfarin (37). Diflunisal also causes a 25-30% increase in hydrochlorothiazide plasma concentrations and a decrease in its urinary excretion. Although the consequences of this interaction are not known, diflunisal does antagonize hydrochlorothiazide uric acid retention (22).

## 6. METHODS OF ANALYSIS

### 6.1 Identification Tests

The USP XXI lists two identification tests for diflunisal. These are an infrared spectrum of the sample in a mineral oil mull and an ultraviolet spectrum of the sample in N/10 methanolic hydrochloric acid. In both cases comparison with a reference sample is required (38).

Supporting evidence for identification can be obtained by thin layer chromatography (section 6.6.1), melting point or differential scanning calorimetry (section 2.1.3) and complexation with ferric ion (section 6.5).

### 6.2 Titrimetric Assay

Diflunisal can be determined by potentiometric titration with aqueous N/10 sodium hydroxide employing a methanol:water (8:1) solvent and a conventional glass/saturated calomel electrode pair.

### 6.3 Ultraviolet Spectrophotometric Analysis

Diflunisal has an absorbance maximum at 315 nm in N/10 methanolic hydrochloric acid characteristic of a salicylate. Comparison with a reference standard at this absorbance maximum commonly is used to quantitate the compound.

A spectrophotometric method has been used as a tentative method of assessing stability. The principal degradation products, 4(2',4'-difluorophenyl)phenol and 3(2',4'-difluorophenyl)benzoic acid show negligible absorbance above 300 nm. The diflunisal descarboxy-diflunisal ester (see section 4.1) interferes with the determination at 315 nm. This compound can be removed by a preliminary solvent extraction at alkaline pH values prior to analysis (39).

### 6.4 Fluorescence Analysis

In pH 7.0 phosphate buffer, diflunisal exhibits a fluorescence maximum at 420 nm and two excitation maxima at 264 nm and 310 nm. Excitation at 310 nm appears to produce

a slightly higher fluorescence emission at 420 nm than excitation at 264 nm. Maximum fluorescence intensity is observed above pH 5.0 where diflunisal is present as a singly charged anion. The detection limit is about 1 ng diflunisal/mL. When other salicylates which interfere with the assay are present in the sample, a chromatographic method is required (13).

### 6.5 Ferric Nitrate Colorimetric Assay

The reaction of salicylates with ferric ion to form a violet complex (40) has been utilized to determine diflunisal in tablets (21). The sample is dispersed in ethanol/water (3:1), solids removed by centrifugation and an aliquot reacted with a 1% aqueous solution of ferric nitrate containing 0.5% nitric acid. The absorbance at 550 nm is linear with concentration from the detection limit (1  $\mu\text{g/mL}$ ) to at least 100  $\mu\text{g/mL}$ . None of the known degradation products of diflunisal interfere (39).

### 6.6 Chromatographic Analysis

#### 6.6.1 Thin Layer Chromatography

A variety of solvent systems for thin layer chromatography of diflunisal have been developed (13, 21, 23). Detection of diflunisal on the dried plate may be accomplished by: (a) quenching of the fluorescent indicator under short wavelength UV light, (b) native blue-violet fluorescence of diflunisal under long wavelength UV light or (c) the formation of a violet spot when the plate is sprayed with a 1% solution of ferric nitrate containing 0.5% nitric acid. A summary of the TLC systems which have been reported is given below.

System I (13)	Type of Sample:	Bulk chemical
	Developing Solvent:	Benzene:dioxane:glacial acetic acid (85:10:5)
	Absorbent:	Silica gel F254
	Identification:	Spot with $R_f$ of 0.55, fluorescence quenching
System II (23)	Type of Sample:	Bulk chemical
	Developing Solvent:	1,2-Dichloroethane: methanol:100% formic acid (97:1:2)
	Absorbent:	Silica gel F254
	Identification:	Spot with $R_f$ of 0.12, fluorescence quenching
System III (23)	Type of Sample:	Urine containing C14-diflunisal

	Developing Solvent:	Butanol:glacial acetic acid:water (4:1:1)
	Absorbent:	Silica gel F254
	Identification:	Radioactive spot at Rf 0.91
System IV (23)	Type of Sample:	Urine containing C14- diflunisal
	Developing Solvent:	Methanol:butanol (12:8)
	Absorbent:	Silica gel F254
	Identification:	Radioactive spot at Rf 0.89
System V (23)	Type of Sample:	Urine containing C14- diflunisal
	Developing Solvent:	n-Propanol:1N NH <sub>4</sub> OH (15:5)
	Absorbent:	Silica gel F254
	Identification:	Radioactive spot at Rf 0.95
System VI (23)	Type of Sample:	Urine containing C14- diflunisal
	Developing Solvent:	Ethyl acetate: ethanol:1N NH <sub>4</sub> OH (85:10:5)
	Absorbent:	Silica gel F254
	Identification:	Radioactive spot at Rf 0.16
System VII (23)	Type of Sample:	Urine containing C14- diflunisal
	Developing Solvent:	Diethyl ether:glacial acetic acid (98.5:1.5)
	Absorbent:	Silica gel F254
	Identification:	Radioactive spot at Rf 0.93
System VIII (23)	Type of Sample:	Urine containing C14- diflunisal
	Developing Solvent:	Benzene:glacial acetic acid (98.5:1.5)
	Absorbent:	Silica gel F254
	Identification:	Radioactive spot at Rf 0.17

### 6.6.2 High Performance Liquid Chromatography

Several methods have been reported which are suitable for: (a) separation of diflunisal from process impurities and potential degradation products (20,21,41), (b) analysis of diflunisal-containing tablets (20,42,43) and (c) determination of diflunisal and its metabolites in biological fluids (44-51). Reversed-phase methods, using an aqueous buffer and one or more organic solvent modifiers to achieve the desired separation, are the most common. Ion pair chromatography using pairing ions such as tetramethylammonium and tetrabutylammonium has given asymmetric peaks and, consequently, poor detection limits for diflunisal (21,23). A summary of the HPLC systems reported is given below.

System I (20)	Type of Sample:	Bulk chemical
	Column:	10 $\mu$ m uBondapak C18 300 x 3.9 mm
	Mobile Phase:	65% A, 35% B A = acetonitrile: methanol:water: glacial acetic acid (20:25:55:2) B = acetonitrile
	Temperature:	40°C
	Flow Rate:	2.4 mL/min
	Detection:	UV at 254 nm
System II (41)	Type of Sample:	Bulk chemical
	Column:	10 $\mu$ m uBondapak C18 300 x 3.9 mm
	Mobile Phase:	Acetonitrile:water: methanol:glacial acetic acid (10:23:55:2)
	Temperature:	40°C
	Flow Rate:	1.5 mL/min
	Retention Time:	18 min
System III (21)	Detection:	UV at 254 nm
	Type of Sample:	Bulk chemical
	Column:	10 $\mu$ m LiChrosorb RP-8, 250 x 4.6 mm
	Mobile Phase:	70% methanol in 0.5% aqueous acetic acid
	Temperature:	45°C
	Flow Rate:	3.0 mL/min

	Retention Time:	2 min
	Detection:	UV at 254 nm
System IV (21)	Type of Sample:	Bulk Chemical
	Column:	10 $\mu$ m Spherisorb ODS 250 x 4.6 mm
	Mobile Phase:	Linear gradient from 30% to 100% B in 20 min. A = 0.005M Tris/ citrate (pH 7.5) B = methanol
	Temperature:	40°C
	Flow Rate:	2.5 mL/min
	Retention Time:	5 min
	Detection:	UV at 254 nm
System V (21)	Type of Sample:	Bulk chemical
	Column:	10 $\mu$ m Spherisorb ODS 250 x 4.6 mm
	Mobile Phase:	60% methanol in aqueous 0.01M Tris/ citrate (pH 3.3)
	Temperature:	40°C
	Flow Rate:	2.0 mL/min
	Retention Time:	2.5 min
	Detection:	UV at 254 nm
System VI (43)	Type of Sample:	Tablets
	Column:	5 $\mu$ m Ultrasphere IP 250 x 4.6 mm
	Mobile Phase:	50% methanol in aqueous 0.001M 18- crown-6 containing 0.05M citrate (pH 5.5)
	Temperature:	40°C
	Flow Rate:	1.5 mL/min
	Retention Time:	6 min
	Detection:	UV at 240 nm
System VII (20)	Type of Sample:	Tablets
	Column:	10 $\mu$ m uBondapak C18 300 x 3.9 mm
	Mobile Phase:	Acetonitrile:formic acid:water (29:1:70)
	Temperature:	40°C
	Flow Rate:	2.0 mL/min
	Retention Time:	10 min

	Detection:	UV at 254 nm
System VIII (42)	Type of Sample:	Tablets
	Column:	10 $\mu$ m uBondapak C18 300 x 3.9 mm
	Mobile Phase:	40% acetonitrile, 1% formic acid in aqueous 0.005M sodium dodecylsulfate
	Temperature:	30°C
	Flow Rate:	2.0 mL/min
	Retention Time:	10 min
	Detection:	UV at 240 nm
System IX (44)	Type of Sample:	Urine or blood plasma
	Column:	10 $\mu$ m uBondapak C18 300 x 3.9 mm
	Mobile Phase:	acetonitrile:0.01M citrate buffer (pH 3.0) (3:7)
	Flow Rate:	2.0 mL/min
	Retention Time:	10.1 min. (diflunisal) 6.0 min (diflunisal ester glucuronide) 3.2 min (diflunisal ether glucuronide)
	Detection:	UV at 254 nm Fluorescence: $\lambda_{ex}$ = 235 nm, $\lambda_{em}$ = 370 nm
System X (45)	Type of Sample:	Urine or blood plasma
	Column:	10 $\mu$ m uBondapak C18 300 x 3.9 mm
	Mobile Phase:	55% B in aqueous 0.1% phosphoric acid B = acetonitrile: methanol (3:1)
	Temperature:	40°C
	Flow Rate:	2.0 mL/min
	Retention Time:	5.3 min
	Detection:	UV at 275 nm
System XI (46)	Type of Sample:	Blood plasma
	Column:	10 $\mu$ m uBondapak C18 300 x 3.9 mm
	Mobile Phase:	Methanol: KH <sub>2</sub> PO <sub>4</sub> buffer (0.03M, pH 7.0) (3:2)

	Temperature:	Not given
	Flow Rate:	Not given
	Retention Time:	Not given
	Detection:	UV at 254 nm
System XII (47)	Type of Sample:	Blood plasma
	Column:	5 $\mu$ m LiChrosorb RP-8, 150 x 4.6 mm
	Mobile Phase:	50% methanol in aqueous 0.01M tetra methylammonium hydro- gen sulfate, with 0.01M Tris
	Temperature:	32°C
	Flow Rate:	1.4 mL/min
	Retention Time:	4.2 min
	Detection:	UV at 254 nm
System XIII (48)	Type of Sample:	Urine or blood plasma
	Column:	5 $\mu$ m Spherisorb ODS, 250 x 4.6 mm
	Mobile Phase:	50% methanol, 33.3% aqueous 0.1M phos- phoric acid, 16.7% tetrahydrofuran. Adjust to pH 3.0 with sodium hydroxide.
	Temperature:	35°C
	Flow Rate:	1.0 mL/min
	Retention Time:	7 min
	Detection:	UV at 254 nm
System XIV (49)	Type of Sample:	Urine or blood plasma
	Column:	5 $\mu$ m Ultrasphere ODS 250 x 4.6 mm
	Mobile Phase:	64% methanol in aqueous 0.05M sodium monobasic phosphate, adjusted to pH 3.0 with sulfuric acid
	Temperature:	50°C
	Flow Rate:	1.0 mL/min
	Retention Time:	8 min
	Detection:	Fluorescence: $\lambda_{ex}$ = 315 nm, $\lambda_{em}$ = 389 nm
System XV (50)	Type of Sample:	Urine or blood plasma
	Column:	5 $\mu$ m Hypersil ODS,



		150 x 4.6 mm
Mobile Phase:		2% acetic acid: isopropanol:ethyl acetate (55:25:20) in aqueous 0.08M potassium nitrate
Temperature:		Ambient
Flow Rate:		1.3 mL/min
Retention Time:		6 min
Detection:		UV at 254 nm
System XVI (51)	Type of Sample:	Urine
	Column:	10 $\mu$ m Hypersil ODS, 300 x 4.6 mm
	Mobile Phase:	Linear gradient from 40% to 100% B in 25 min. A = 30% methanol in 2% aqueous acetic acid B = 65% methanol in 2% aqueous acetic acid
	Temperature:	Ambient
	Flow Rate:	2 mL/min
	Retention Time:	10.1 min (diflunisal ether glucuronide) 15.4 min (diflunisal ester glucuronide) 25.8 min (diflunisal)
	Detection:	UV at 254 nm

### 6.6.3 Gas Chromatography

Reported GC methods for the analysis of diflunisal involve prior conversion to the methyl ester with methanol/boron trifluoride (52), diazomethane (53) or trimethyl-anilinium hydroxide (54). Practical details involved in using these and other derivatization reagents for carboxylic acids have been compiled (55). A summary of the GC methods which have been reported is given below.

System I (56)	Type of Sample:	Bulk chemical
	Column:	6 ft. x 2 mm (i.d.) glass column packed with 10.5% ethylene glycol adipate polyester and 1.75% phos- phoric acid on 110/120 mesh Anakrom HBS
	Carrier Gas:	N <sub>2</sub>
	Flow Rate:	25 mL/min
	Column Temperature:	190°C

	Retention Time:	24 min (methyl ester)
	Detector:	FID
System II (56)	Type of Sample:	Urine or blood plasma
	Column:	6 ft. column packed with 1% QF-1 on 100/120 mesh GasChrom Q
	Flow Rate:	50 mL/min
	Column Temperature:	160°C
	Retention Time:	4.2 min (methyl ester)
	Detector:	FID
System III (52)	Type of Sample:	Blood plasma
	Column:	15 m x 0.32 mm (i.d.) capillary column coated with SE-52, 0.4-0.45 $\mu$ m film thickness
	Carrier Gas:	He
	Flow Rate:	3 mL/min
	Column Temperature:	Programmed from 85°C at 20°C/min to 180°C, hold for 3 min, then at 3.5°C/min to 240°C
	Detector:	FID
System IV (53)	Type of Sample:	Blood plasma
	Column:	5% FFAP on Chromosorb WHP
	Carrier Gas:	N <sub>2</sub>
	Flow Rate:	45 mL/min
	Column Temperature:	275°C
	Retention Time:	5 min (methyl ester)
	Detector:	FID

### 6.7 Determination in Biological Fluids

The fluorometric assay for intact diflunisal in plasma reported by Tocco et al. (23) requires acidification of the sample with perchloric acid, extraction with chloroform and back-extraction of the organic phase with pH 8.0 phosphate buffer prior to the fluorescence measurement. This procedure is not applicable to urine samples because of interference from the labile ester glucuronide of diflunisal. Plasma samples do not pose a problem in this regard because the glucuronides are present at low concentration and are poorly extracted into chloroform. A com-

parison of the fluorometric method with an HPLC method using UV detection (50) demonstrated good agreement between the two methods. The detection limit for diflunisal in plasma by the fluorometric method is about 1  $\mu\text{g/mL}$  (50). Several HPLC assay methods for plasma and urine samples are listed in section 6.6.2.

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# BACLOFEN

Satinder Ahuja

*CIBA-GEIGY Corporation  
Suffern, New York*

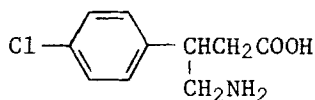
1	Description	528
1.1	Introduction	528
1.2	Formula, Name, Formula Weight	528
1.3	Appearance, Color, Odor, Taste	528
2.	Physical Properties	528
2.1	Ultraviolet Spectroscopy	528
2.2	Infrared Spectroscopy	530
2.3	Nuclear Magnetic Resonance Spectroscopy	530
2.4	Mass Spectrometry	533
2.5	Optical Rotation/Circular Dichroism	533
2.6	Melting Range	533
2.7	Differential Scanning Calorimetry	533
2.8	Thermogravimetry	535
2.9	X-Ray Powder Diffraction	535
2.10	Dissociation Constant	535
2.11	Solubility	535
2.12	Water Absorption	536
2.13	Distribution Coefficient	536
3.	Synthesis	536
4.	Stability	538
4.1	Solid State Stability	538
4.2	Solution Stability	538
5.	Pharmacokinetics, Metabolism, and Activity	539
5.1	Absorption/Excretion	539
5.2	Blood Plasma Concentrations	539
5.3	Tissue Distribution	540
5.4	Elimination	540
5.5	Transformation	540
5.6	Enzyme Induction, Protein Binding	541
5.7	Activity of Enantiomers	543
6.	Analytical Methodology	543
6.1	Titrimetry	543
6.2	Gas Chromatography	543
6.3	High Pressure Liquid Chromatography	544
6.4	Thin-Layer Chromatography	546
6.5	Colorimetry	546
6.6	Mass Fragmentography	547
7.	Toxicological Studies	547
	References	549

## 1. Description

### 1.1 Introduction

Baclofen is a synthetic antispastic agent or muscle relaxant.

### 1.2 Formula, Name, Formula Weight



Baclofen

Formula Weight: 213.67

$C_{10}H_{12}ClNO_2$

Baclofen has been described by the following chemical names:

- (i) 4-Amino-3(p-chlorophenyl) butyric acid
- (ii)  $\beta$ -(Aminomethyl)-4-chlorobenzenepropionic acid
- (iii)  $\beta$ -(Aminomethyl)-p-chlorohydrocinnamic acid
- (iv)  $\gamma$ -Amino- $\beta$ -(p-chlorophenyl)butyric acid
- (v)  $\beta$ -(4-Chlorophenyl) GABA

Other names: Lioresal, Ba 34,647

### 1.3 Appearance, Color, Odor, Taste

Baclofen is a white to off-white, virtually odorless, crystalline powder with slightly bitter taste.

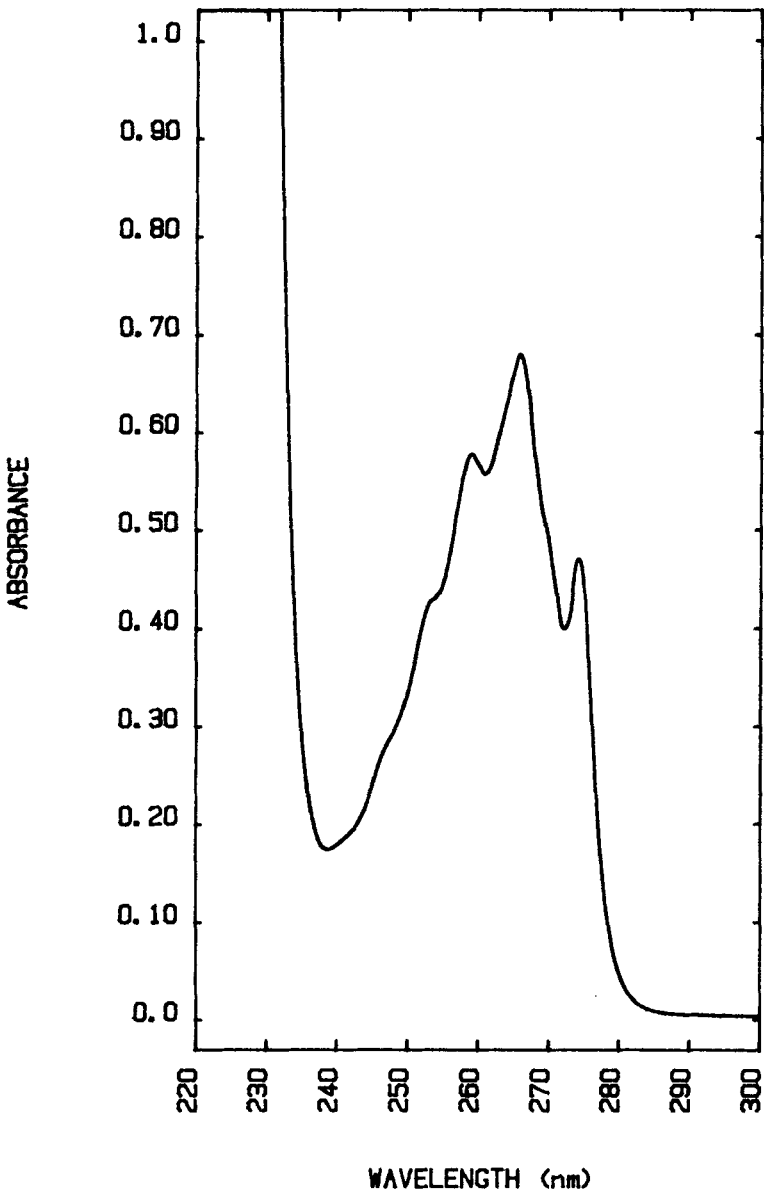
## 2. Physical Properties

### 2.1 Ultraviolet Spectroscopy

The ultraviolet absorption spectrum of baclofen exhibits characteristic aromatic absorption in the region 258-274 nm (1). The bands arise from  $\pi \rightarrow \pi^*$  transition of the electrons in the phenyl ring (aromatic). The observed spectrum of baclofen in 0.1N HCl (Figure 1) is comparable to that of the aromatic amino acids. It has the following A(1%, 1 cm) values at the indicated  $\lambda_{max}$ :

FIGURE 1

Ultraviolet Absorption Spectrum of Baclofen in 0.1N HCl


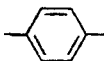




$\lambda_{\max}$	$A(1\%, 1 \text{ cm})$
274 nm	7.9
266 nm	11.3
258 nm	9.6

## 2.2 Infrared Spectroscopy

The infrared absorption spectrum of baclofen in Nujol (Figure 2) exhibits the following bands which are consistent with its structure:

Wavenumber ( $\text{cm}^{-1}$ )	Assignment
2650, 2180	$-\text{NH}_2$ *
1610, 1580	$-\text{NH}_2$ *, 
1530	$-\text{COOH}^*$
1400	$-\text{COOH}^*$
1100	$-\text{C}-\text{Cl}$
1020, 830	

\*Probably occurs as zwitterion

## 2.3 Nuclear Magnetic Resonance Spectroscopy:

The NMR spectrum of baclofen in deuterated trifluoroacetic acid (Figure 3) is consistent with its structure:

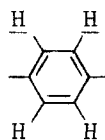
<u>Chemical Shift</u> (ppm)	<u>Multiplicity</u>	<u>Relative Integral</u> <u>of Protons</u>	<u>Assignment</u>
2.7 - 3.2	Multiplet	2	$-\text{CH}-\text{CH}_2-\text{COOH}$
3.2 - 3.9	Multiplet	3	$-\text{CH}-\text{CH}_2-\text{NH}_2$
6.8 - 7.7	Quartet	-	 + Exchangeable Protons
10.9	Singlet	-	Exchangeable Protons

FIGURE 2

Infrared Absorption Spectrum of Baclofen in Nujol

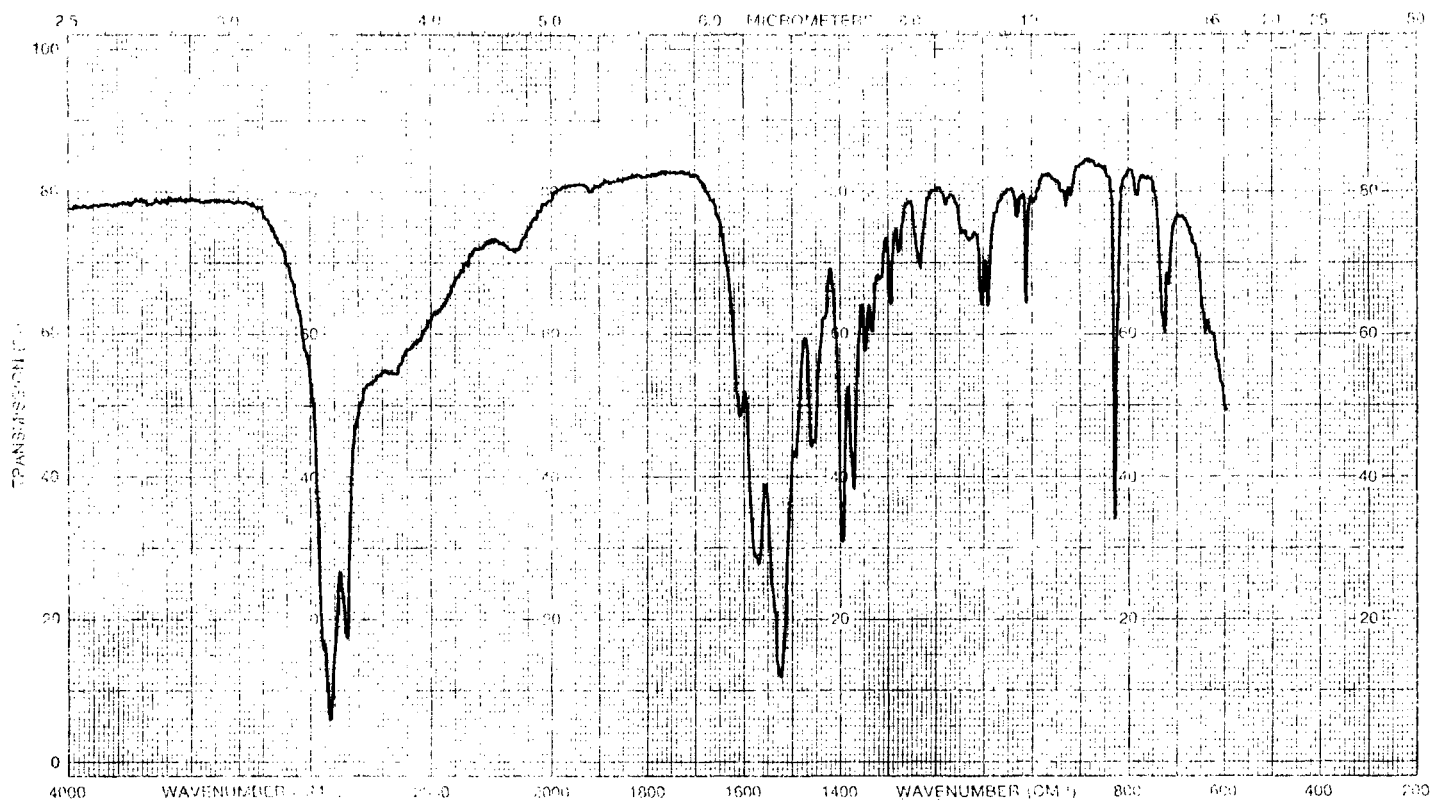
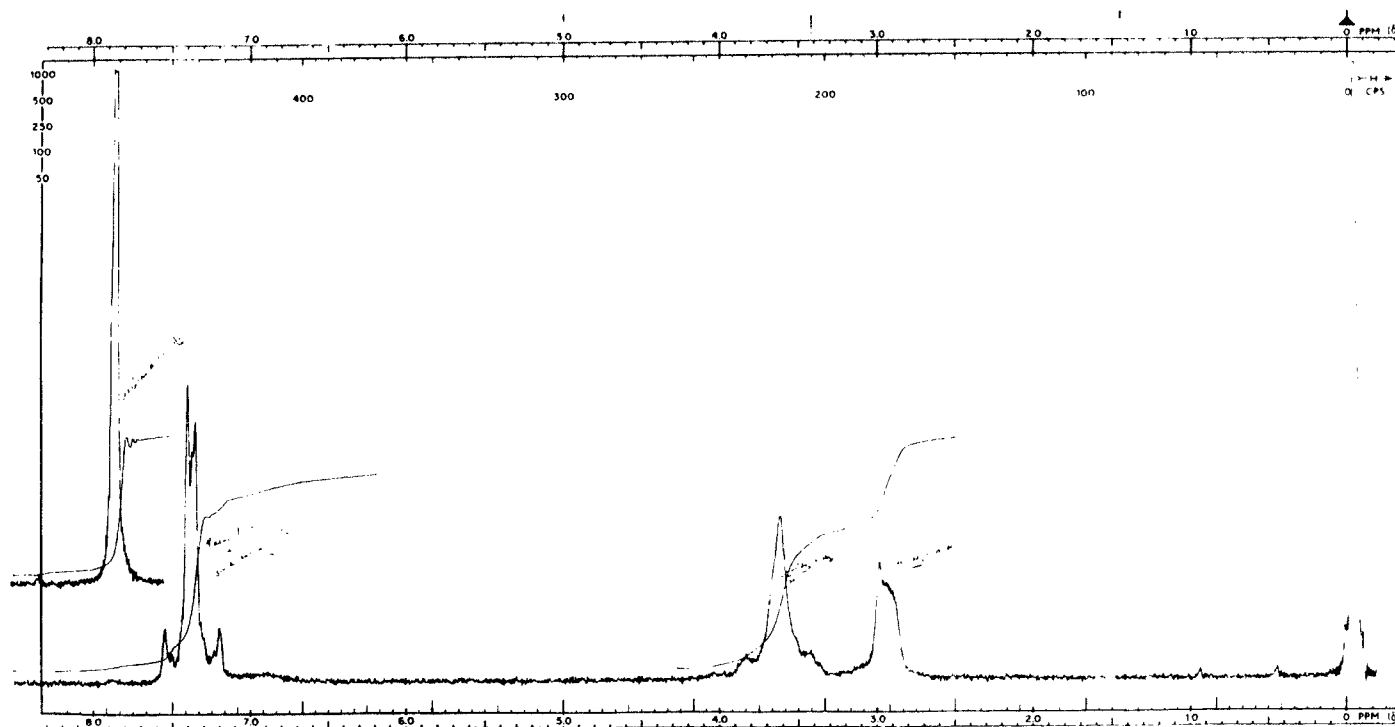


FIGURE 3  
NMR Spectrum of Baclofen in Trifluoroacetic Acid



## 2.4 Mass Spectrometry

The mass spectrum (Figure 4) is compatible with the indicated structure of baclofen and shows the following fragmentation pattern:

m/e	Structure
213	M+
195	$\left[ \text{Cl}-\text{C}_6\text{H}_4-\text{C} \begin{array}{l} \text{H} \\   \\ \text{CH}_2 \\   \\ \text{C}=\text{O} \\   \\ \text{CH}_2 \\   \\ \text{NH} \end{array} \right]^+$
138	$\left[ \text{Cl}-\text{C}_6\text{H}_4-\text{C} \begin{array}{l} \text{H} \\   \\ = \text{CH}_2 \end{array} \right]^+$
103	$\left[ 138 \text{ minus Cl } \right]^+$
77	$\left[ \text{C}_6\text{H}_5 \text{ minus H } \right]^+$

## 2.5 Optical Rotation/Circular Dichroism

No optical rotation ( $[\alpha]_D^{20}=0^\circ$ ) is observed with aqueous (0.3%), acidic or basic solutions. No circular dichroism is observed.

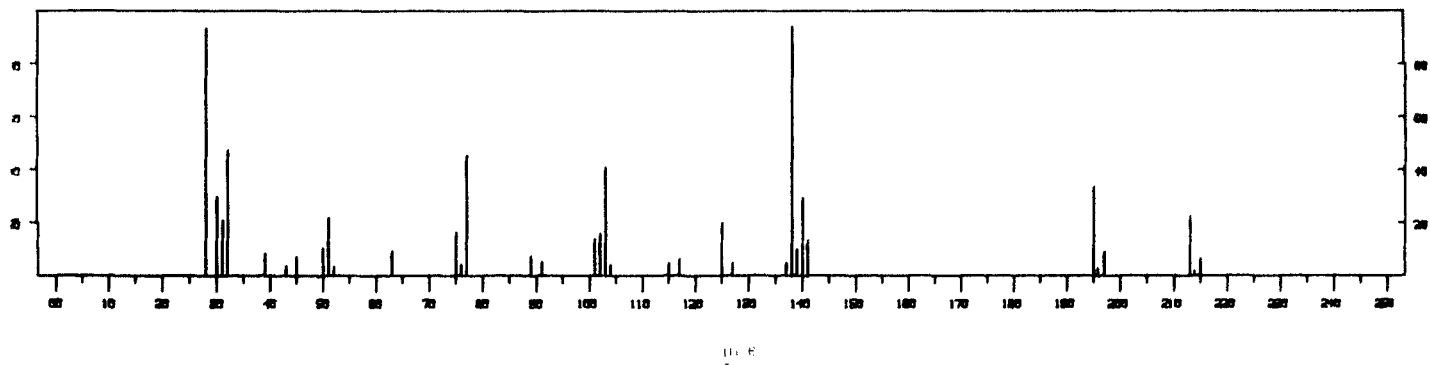
## 2.6 Melting Range

The melting range (192-193°C) of baclofen can vary due to lactam formation with a concomitant loss of water.

## 2.7 Differential Scanning Calorimetry

No reliable purity value can be determined since baclofen melts with decomposition.

FIGURE 4  
Mass Spectrum of Baclofen



2.8 Thermogravimetry

Thermogravimetric analysis generally gives a weight loss of <1% between 30 - 155°C.

2.9 X-ray Powder Diffraction

Baclofen shows the following intense x-ray lines, 2 $\theta$ : 5.7, 17.5, 19.0, 22.0, 23.4, 26.3, and 29.8. Baclofen is orthorhombic P2<sub>1</sub>2<sub>1</sub>2<sub>1</sub>, a = 6.373, b = 7.318, c = 25.699 Å and Z = 4 with the final R factor of 0.040 for 1183 reflections (2).

2.10 Dissociation Constant

The pK<sub>a</sub> values in water (5.0 x 10<sup>-3</sup> moles/l) at 20°C are as follows:

$$\text{pK}_{a1} = 3.87 \pm 0.1 \text{ (carboxyl group)}$$

$$\text{pK}_{a2} = 9.62 \pm 0.1 \text{ (amino group)}$$

2.11 Solubility

The equilibrium solubility values in various solvents, at the indicated temperature, are given in Table I:

TABLE I

Solubility of Baclofen

<u>Solvent</u>	<u>Temperature</u>	<u>mg/ml</u>
Water (pH 7.6)	23°C	4.3
Methanol	RT	0.045
Ethanol	RT	0.024
Chloroform	RT	0.014
Dimethylformamide	RT	0.008
Acetonitrile	RT	0.004
Phosphate Buffer (pH 7.4)	RT	5.0
0.1N HCl	RT	>20
0.1N NaOH	RT	>20

### 2.12 Water Absorption

The uptake of water by baclofen at various relative humidities at 40°C, as determined by Karl Fischer method, is given in Figure 5 (3).

### 2.13 Distribution Coefficient

Distribution coefficients in various organic phases vs pH 7.4 at the indicated temperature are given in Table II (4).

TABLE II

Distribution Coefficients of Baclofen

<u>Organic Phase</u>	<u>Aqueous Phase *</u>	<u>Temperature °C</u>	$k = \frac{C_{org.}}{C_{aq}}$
Butanol	pH 7.4	25	0.67
Octanol	pH 7.4	23	0.11
Methylene-Chloride	pH 7.4	23	0.003
Ether	pH 7.4	20	0.003
Benzene	pH 7.4	23	0.003
Hexane	pH 7.4	23	0

\*Phosphate buffer

### 3. Synthesis

The last step in baclofen synthesis can be carried out in several different ways. One process utilizes the reaction between  $\beta$ -(p-chlorophenyl)-glutarimide and bromine in an excess of aqueous sodium hydroxide at low temperatures. An alternate process is carried out in sodium hydroxide-sodium hypochlorite solution at room temperature:

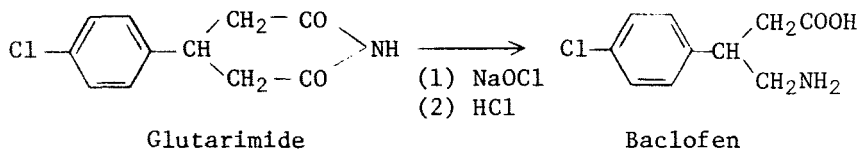
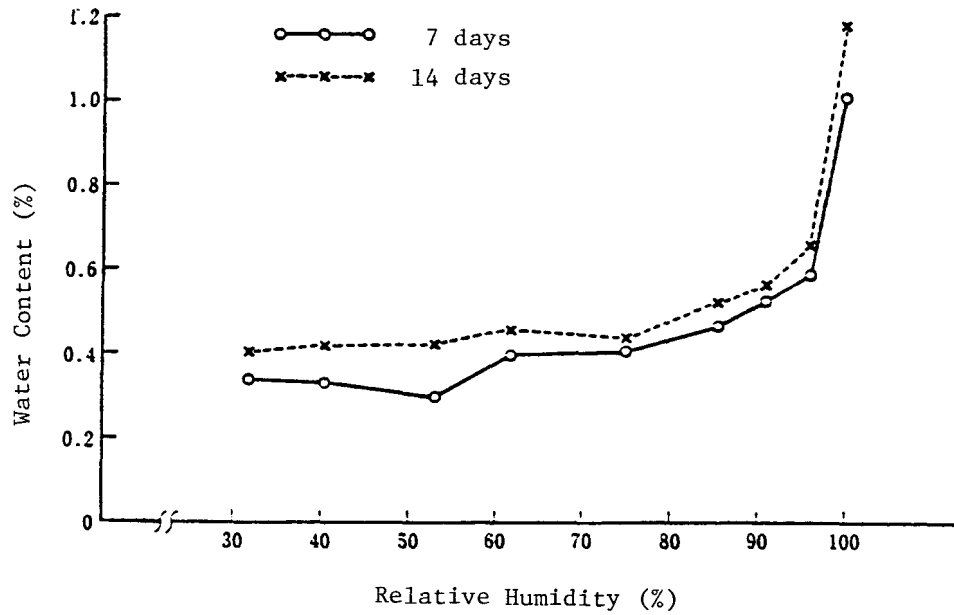


FIGURE 5

Water Absorption Isotherm of Baclofen (3)

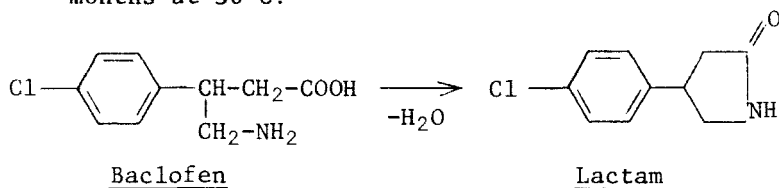




#### 4. Stability

##### 4.1 Solid State Stability

Baclofen is a stable compound under normal storage conditions. At high temperatures, e.g. 50°C, formation of the corresponding lactam [4-4(4-chlorophenyl)-2-pyrrolidone] is observed (4, 5). Above 160°C, decomposition proceeds at a fast rate. Practically no change is observed in the samples stored for 12 months at 35°C or 6 months at 50°C.



##### 4.2 Solution Stability

The lactam formation in aqueous solutions at high temperatures is dependent on pH of the medium. Loss of baclofen can be determined by the colorimetric reaction with ninhydrin (6). Based on studies performed at 90°C, 80°C and 70°C, the pseudo first order rate constants from pH 2 to 12 are given in Table III:

TABLE III

Solution Stability of Baclofen Active Ingredient

pH	$k \times 10^5 \text{ (days}^{-1}\text{)}$	t10%, 25°C
2	8.41	3.4 years
4	5.07	5.7 years
6	3.02	9.6 years
7	2.57	11.2 years
9	14.4	2.0 years
10	14.5	2.0 years
11	11.5	2.5 years
12	4.21	6.9 years

The k values for pH 10, 11 and 12 are estimates based on k value at 80°C, assuming the same activation energy as pH 7 and 9 and the same temperature effect. In 2N hydrochloric acid and in 2N sodium hydroxide, the reaction rates are 0.

A preliminary kinetic study with a more selective HPLC method (ion-exchange method) shows that baclofen solutions in 0.07M HNO<sub>3</sub> or 0.1M NaOH follow pseudo-first order reaction for less than the first half-life (11).

## 5. Pharmacokinetics, Metabolism and Activity

Since baclofen is amphoteric, it occurs as zwitterion at pH ~7. Distribution data in octanol/water (Section 2.13) suggest baclofen is slightly lipophilic.

### 5.1 Absorption/Excretion

Since 90% of baclofen is excreted in urine of rats, it is apparent this percentage must have been absorbed to be excreted in urine; the rest of the dose is excreted in feces. The same ratio is found when the drug is administered i.v. Similar behavior is observed in dog and man. Evidence of absorption in man is derived from bioavailability studies employing analysis of unchanged baclofen in urine. In a study of experimental formulations, five subjects ingested 20 mg of baclofen as tablets on two occasions (8). Renal excretion of unchanged baclofen ranges from 49.5 to 82.8% of dose in 24 hours, based on G.C. analysis, with 21.7 - 37.5% excreting within four hours after dosage. Maximum plasma concentrations are observed at 1 - 2 hours after dosage. No statistically significant difference is found as a result of fasting, either in the extent or the rate of excretion.

### 5.2 Blood Plasma Concentrations

Plasma samples from five subjects ingesting a 10-mg to 40-mg oral dose of <sup>14</sup>C-labeled baclofen show maximum concentrations ranging from 0.1 to 0.8 µg/ml, by inverse isotope dilution method, two hours after dosing. The concentrations decrease to less than 0.2 µg/ml at the eighth hour, with a half-life of approximately 2.5 - 4.0 hours.

Gas chromatographic analysis of plasma and cerebrospinal fluid samples from two multiple sclerosis patients on the fifth day of baclofen therapy (10 mg t.i.d.) shows plasma concentrations of 203 - 278 ng/ml for one patient and 91 - 179 ng/ml for the other. The concentrations in cerebrospinal fluid are 11 - 13 ng/ml and 8 - 25 ng/ml, respectively.

### 5.3 Tissue Distribution

The distribution of radioactivity in rat and mouse tissues after intravenous injection of  $^{14}\text{C}$ -labeled baclofen has been extensively studied. Twenty different tissues were analyzed from three rats sacrificed after 10 mg/kg intravenous dosage. At five minutes after injection all of the tissues show measurable radioactivity, with the highest concentrations being found in kidney (149  $\mu\text{g/g}$ ) and liver (27  $\mu\text{g/g}$ ). At six hours after dosing, the average value in kidney is 2.42  $\mu\text{g/g}$ ; all other tissues are less than 1  $\mu\text{g/g}$ . Small amounts of radioactivity are still detectable in many tissues at 72 hours, but not at 168 hours after dosage. The half-life of radioactivity in tissues of three rats was measured after 5 mg/kg i.v. of  $^{14}\text{C}$ -labeled baclofen (8). The decrease in radioactivity is approximately exponential during the first six hours after dosage. During this period half-life values for radioactivity in blood and most other tissues are  $2 \pm 0.5$  hours. Brain, nerve tissue and testes show values about twice as great. Similar distribution of baclofen is found in mouse tissues (8).

### 5.4 Elimination

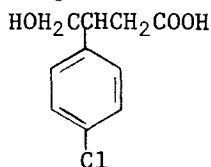
Baclofen is readily eliminated by renal excretion, e.g. 55 - 92% radioactivity is excreted in urine by five subjects receiving a 10 mg to 40 mg oral dose of  $^{14}\text{C}$ -labeled baclofen. The unchanged drug in urine, as measured by an inverse isotope dilution method, comprises 94 - 96% of the total radioactivity in the urine.

### 5.5 Transformation

The concentration of metabolites is at a maximum between 2 and 4 hours after oral dosage, followed by a decrease to less than 0.05  $\mu\text{g/ml}$  at 24 hours. Analysis of urine from these subjects

shows that only 3 - 6% of the dose is excreted renally as metabolites, with the major portion being excreted in urine as unchanged baclofen.

A metabolite of baclofen is identified as  $\beta$ -(hydroxymethyl)-p-chlorohydrocinnamic acid (metabolite A). Inverse isotope dilution analysis shows the presence of this compound in plasma from rats given 5 mg/kg of  $^{14}\text{C}$ -labeled baclofen by intravenous injection.



Metabolite A

#### 5.6 Enzyme Induction, Protein Binding

No marked difference from control values is apparent when urine samples from six rats given 0.625% baclofen in feed for 12 months are analyzed for ascorbic acid. The effect of baclofen administration on the duration of pharmacological response of rats to hexobarbital and to zoxazolamine has been also examined. Groups of six rats were given either 5 mg/kg or 25 mg/kg of baclofen twice daily for three consecutive days. On the fourth day, the duration of sleep or paralysis was measured after an intraperitoneal injection of zoxazolamine or sodium hexobarbital. Pretreatment with baclofen does not change the ability of the animals to metabolize hexobarbital or zoxazolamine.

In another study, three groups of rats were given an intravenous 5 mg/kg dose of  $^{14}\text{C}$ -labeled baclofen after pretreatment with ten daily doses of non-radioactive baclofen. The pretreatment doses of either 0, 5.0, or 12.5 mg/kg were administered by intraperitoneal injection. Blood, urine and tissue samples were collected at various times and analyzed for total radioactivity. Blood samples were also analyzed by inverse isotope dilution for baclofen and for metabolite A. For all three groups of rats, the extrapolated initial concentration of metabolite A ranges

from 0.02 to 0.08  $\mu\text{g/ml}$ . No differences are noted in the half-life of radioactivity in tissues, or in the kinetics of urinary excretion, as a result of pretreatment with baclofen. Thin-layer chromatographic examination of urine samples indicates no differences in the amounts of unchanged drug (80 - 85%) or in the amounts and chromatographic behavior of metabolites.

Binding of baclofen to the proteins of human serum is low (9). In vitro measurements over a wide concentration range between 10 ng/ml and 300  $\mu\text{g/ml}$  show that only about 30% is bound. Because of the low extent of binding it is unlikely that displacement from binding sites by another drug would strongly influence the clinical effects of baclofen.

#### 5.7 Activity of Enantiomers

Of the two enantiomers of racemic baclofen, the  $\ell$ -form is much more active than the d-form. Listed in Table IV are results of an animal study performed by Olpe *et al* (10). Depression of different reflexes in cats, and protection against electroshock-induced convulsions in mice are demonstrable for  $\ell$ -baclofen only. However, it must be noted that the toxicity of baclofen also resides mainly with the  $\ell$ -enantiomer.

TABLE IV

#### Activity of Baclofen Enantiomers (10)

<u>Property</u>	<u>(-)-<math>\ell</math>-baclofen</u>	<u>(+)-d-baclofen</u>
Specific rotation (1% solution)	-1.4°	+1.4°
Reflex inhibition (i.v. cat)	100% at 1-3 mg/kg	0% at 30 mg/kg
Protection E-shock (p.o. mouse)	50% at 60 mg/kg	0% at 100 mg/kg
Acute toxicity (p.o. mouse; 1000 mg/kg)	9/10	0/10

## 6. Analytical Methodology

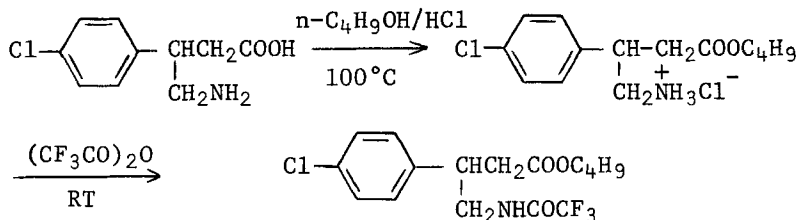
There are several inherent difficulties in developing a suitable method for the analysis of baclofen in various matrices. Baclofen is poorly soluble in most organic solvents and exhibits low partition coefficients into organic solvents. The low absorptivity values for baclofen in the ultraviolet region and interferences from various inert ingredients in the preparation are additional limiting factors. As mentioned earlier, stability studies with the active ingredient in both the solid state and in solution, and with formulations, show that 4-(4-chlorophenyl)-2-pyrrolidone is the major decomposition product. Only those analytical methods that are specific for baclofen in the presence of 4-(4-chlorophenyl)-2-pyrrolidone are discussed below (11).

### 6.1 Titrimetry

Baclofen active ingredient can be titrated either as an acid or as a base. The amino group can be titrated in acetic acid with perchloric acid. The carboxylic acid moiety of baclofen can be titrated in 80% ethylene glycol monomethyl ether (EGME) with sodium hydroxide after reacting the primary amino group with formaldehyde. Titrimetry is a valuable method for determining the purity of baclofen because the glutarimide precursor will not titrate under these conditions. However, due to possible interferences from tablet excipients (11), this method may not be suitable for evaluation of formulations.

### 6.2 Gas Chromatography

Since baclofen has both primary amine and carboxylic acid groups, derivatization of both is necessary prior to gas chromatography (11). The derivatization reaction scheme follows:



The resulting derivative is chromatographed on a Tabsorb column at 200°C. With this method, 4-(4-chlorophenyl)-2-pyrrolidone yields a broad peak which does not interfere with the quantitation of baclofen; however, it is not possible to quantitate 4-(4-chlorophenyl)-2-pyrrolidone by this method.

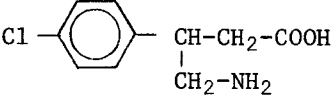
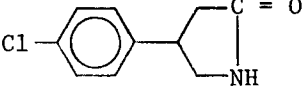
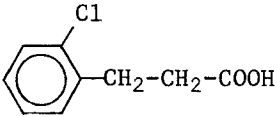
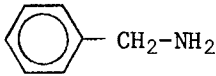
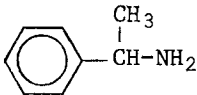
Absorption on charcoal or ion-pair extraction are used for isolating baclofen from biological fluids (12, 13). After elution, baclofen is derivatized and analyzed with an electron capture detector.

### 6.3 High Pressure Liquid Chromatography

Baclofen can be chromatographed on a cation exchange column (11) utilizing 0.1M HNO<sub>3</sub> in 30% methanol as a sample solvent and an acidic mobile phase (0.01M in tetramethyl ammonium nitrate and 0.02M in nitric acid). With this system, 4-(4-chlorophenyl)-2-pyrrolidone elutes before baclofen. The quantitation of the 4-(4-chlorophenyl)-2-pyrrolidone impurity by this method is more accurate than semi-quantitative estimations by thin-layer chromatography. The method is suitable for the analysis of a variety of different tablet formulations of baclofen.

The selectivity of the method for detection of various degradation products is shown in Table V. The data indicate that baclofen is separated from its major degradation compound, 4-(4-chlorophenyl)-2-pyrrolidone (Compound 2). The separation of baclofen from o-chloro-hydrocinnamic acid (Compound 3), a model compound, indicates that compounds resulting from deamination are likely to be separated from baclofen. Similarly, the separations obtained for the other two model compounds, benzylamine (Compound 4) and  $\alpha$ -methylbenzylamine (Compound 5) indicate that the method would be selective for baclofen in the presence of compounds resulting from decarboxylation.

TABLE V  
Selectivity of Ion-Exchange HPLC Method

<u>Compound</u>	<u>Structure</u>	<u>Retention Volume, ml.</u>
1. Baclofen		5.91
2. 4-(4-Chlorophenyl)-2-pyrrolidone		1.97
3. o-Chlorohydrocinnamic acid		1.97
4. Benzylamine		9.84
5. dl-α-Methylbenzylamine		18.6



The results of an accelerated study at 90°C on powdered baclofen tablets suspended in water indicate that the method is specific for baclofen in the presence of degradation products (11). Unknown peaks are observed in the liquid chromatogram; however, 4-(4-chlorophenyl)-2-pyrrolidone is the major decomposition product.

Improved separations are possible with an ion-pair reversed phase liquid chromatographic method (14). "Ion-pairing" between baclofen and pentane sulfonic acid is primarily responsible for the separation of baclofen from the transformation products since they do not have amino group and cannot form an ion-pair.

#### 6.4 Thin-Layer Chromatography

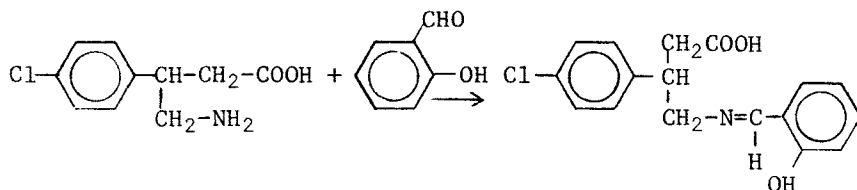
The following TLC systems can be used for chromatographing baclofen:

50 Toluene/40 Ethanol/7 Ammonium Hydroxide  
7 Chloroform/3 Ethyl Acetate  
4 n-Butanol/1 Acetic Acid/1 Water

The glutarimide and the 4-(4-chlorophenyl)-2-pyrrolidone can be resolved with all of these systems; however, the 4 n-Butanol/1 Acetic Acid/1 Water system provides the best resolution for these compounds and degradation compounds formed at trace levels in the preliminary kinetic studies.

#### 6.5 Colorimetry

A method based on the following colorimetric reaction of baclofen with salicylaldehyde can be used for analysis of baclofen tablets (11):



This method can be automated and is suitable for monitoring content uniformity and average content of baclofen tablets. It is selective for baclofen since 4-(4-chlorophenyl)-2-pyrrolidone and the glutarimide do not react with salicylaldehyde.

#### 6.6 Mass Fragmentography

A mass fragmentographic method for determination of baclofen in cerebrospinal fluid has been described (15). The amino acid is chromatographed as its pentafluoropropyl ester pentafluoropropyl amide. Measurement in biological fluids is possible down to a concentration of 5 ng/ml.

#### 7. Toxicological Studies

Oral toxicological testing in male rats gives the following results for baclofen and 4-(4-chlorophenyl)-2-pyrrolidone:

Baclofen Suspension in PEG 300: 228 mg/kg

4-(4-chlorophenyl)-2-pyrrolidone solution in PEG 300: 841 mg/kg

These results suggest that 4-(4-chlorophenyl)-2-pyrrolidone is significantly less toxic than baclofen.

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## **PROFILE SUPPLEMENTS**

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# ACETAMINOPHEN

Humeida A. El-Obeid and  
Abdullah A. Al-Badr

*King Saud University  
Riyadh, Saudi Arabia*

1. Description	552
1.1 Nomenclature	552
1.2 Formulae	552
1.3 Molecular Weight	553
1.4 Elemental Composition	553
1.5 Appearance, Color, Odor, and Taste	553
2. Physical Properties	553
2.1 Melting Point	553
2.2 Solubility	553
2.3 Spectral Properties	553
3. Pharmacokinetics	563
3.1 Absorption and Excretion	563
3.2 Metabolism	564
4. Methods of Analysis	567
4.1 Titrimetric Methods	569
4.2 Electrochemical Methods	569
4.3 Spectrophotometric Methods	570
4.4 Chromatographic Methods	578
References	590

## 1. Description

### 1.1 Nomenclature

#### 1.1.1 Chemical Names

P-Acetamidophenol, p-acetaminophenol, N-acetyl-p-aminophenol, p-hydroxyacetanilide, 4'-hydroxyacetanilide, p-acetylaminophenol, N-(4-hydroxyphenyl) acetamide, N-p-hydroxyphenylacetamide.

#### 1.1.2 Generic Names

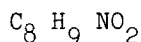
Acetaminophen, Acetophenum, Paracetamol.

#### 1.1.3 Trade Names

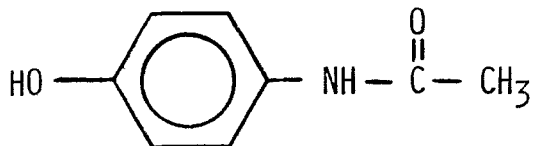
Abensanil, Acamol, Acetalgin, Amadil, Anaflon, Apamide, APAP, Bickie-mol, Calpol, Cetadol, Datriil, Dial-a-gesic, Dirox, Dymadon, Eneril, Febrilix, Finimal, Gelocatil, Hedex, Homoolan, Korum, Lyteca, Naprinol, Nobedon, Pacemo, Panadol, Panets, Paraspen, Parmol, Tabalgin, Tapar, Temlo, Temptra, Tralgon, Tylenol, Valadol.

### 1.2 Formulae

#### 1.2.1 Empirical



#### 1.2.2 Structural



#### 1.2.3 CAS Registry No.

[103-90-2]

#### 1.2.4 Wiswesser Line Notation

QR DMV1

#### 1.3 Molecular Weight

151.16

#### 1.4 Elemental Composition

C, 63.56 %; H, 6.00 %, N, 9.27 %; O, 21.17%.

#### 1.5 Appearance, Color, Odor and Taste

White, odorless, slightly bitter crystalline powder.

### 2. Physical Properties

#### 2.1 Melting Point

169-170.5°.

#### 2.2 Solubility

1 in 70 of water, 1 in 20 of boiling water, 1 in 7 of alcohol, 1 in 13 of acetone, 1 in 50 of chloroform, 1 in 40 of glycerol, 1 in 10 of methyl alcohol and 1 in 9 of propylene glycol; insoluble in ether, soluble in solutions of alkali hydroxides. A saturated solution has a pH of about 6.

#### 2.3 Spectral Properties

##### 2.3.1 Ultraviolet Spectrum

The ultraviolet spectra of acetaminophen in water, acidified water, neutral methanol, acidified methanol and methanol containing sodium methoxide are obtained using Cary, 219 spectrophotometer and are shown in Figures 1 and 2.

In neutral water, acetaminophen shows a major band at 242 nm and a minor band around 280 nm. Addition of acid does not seem to affect either band. (Figure 1).



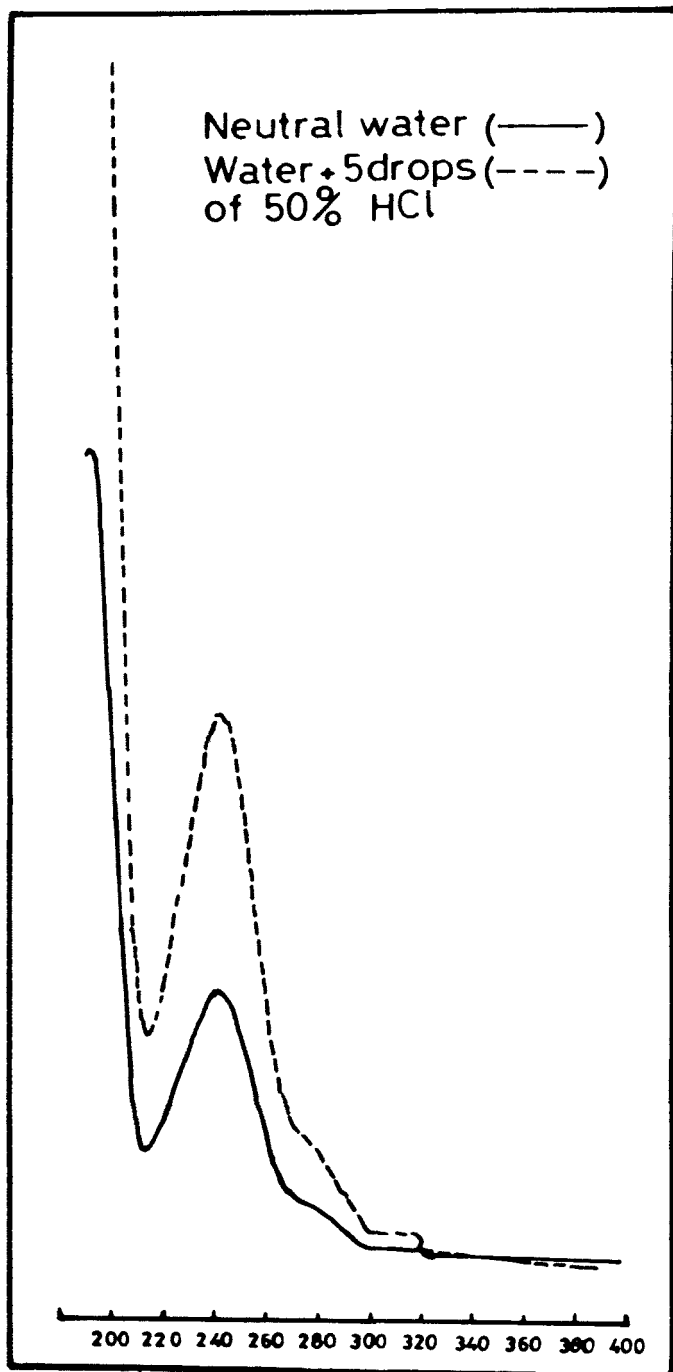


Figure 1 Ultraviolet spectrum of acetaminophen

In neutral methanol the main band appears at 243 nm and its position is not affected by acid. However, the addition of sodium methoxide to the methanolic solution of acetaminophen caused a bathochromic shift from 243 nm to 262 nm. (Figure 2). This red shift is due to the ionization of acetaminophen to p-acetamidophenolate ion.

### 2.3.2 Infrared Spectrum

The infrared spectrum of acetaminophen in KBr disc (Figure 3) is recorded on a Perkin-Elmer spectrophotometer model 580 B. The spectral interpretation is given below:

<u>Frequency (cm<sup>-1</sup>)</u>	<u>Assignment</u>
800-860	C-H out of plane bending of p-substituted benzene ring.
1440 ) 1505 ) 1560 )	Aromatic vibrations
1651	
3160	
3324	N-H stretch

### 2.3.3 <sup>1</sup>H Nuclear Magnetic Resonance (<sup>1</sup>H NMR) Spectrum

The <sup>1</sup>H NMR spectrum of acetaminophen is shown in Figure 4. The drug is dissolved in acetone-d<sub>6</sub> and its spectrum determined on a Varian-T60A NMR spectrometer using TMS as the internal standard.

Assignment of the chemical shifts to the different protons is shown below:

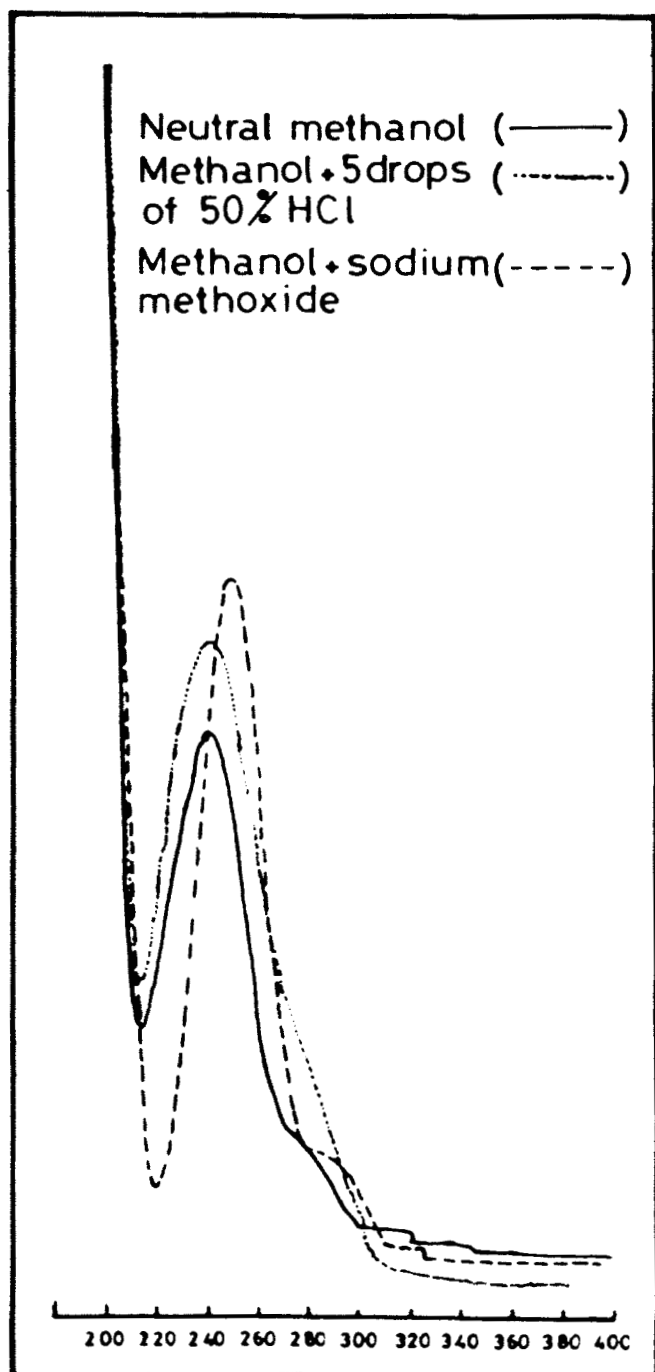


Figure 2 Ultraviolet spectrum of acetaminophen

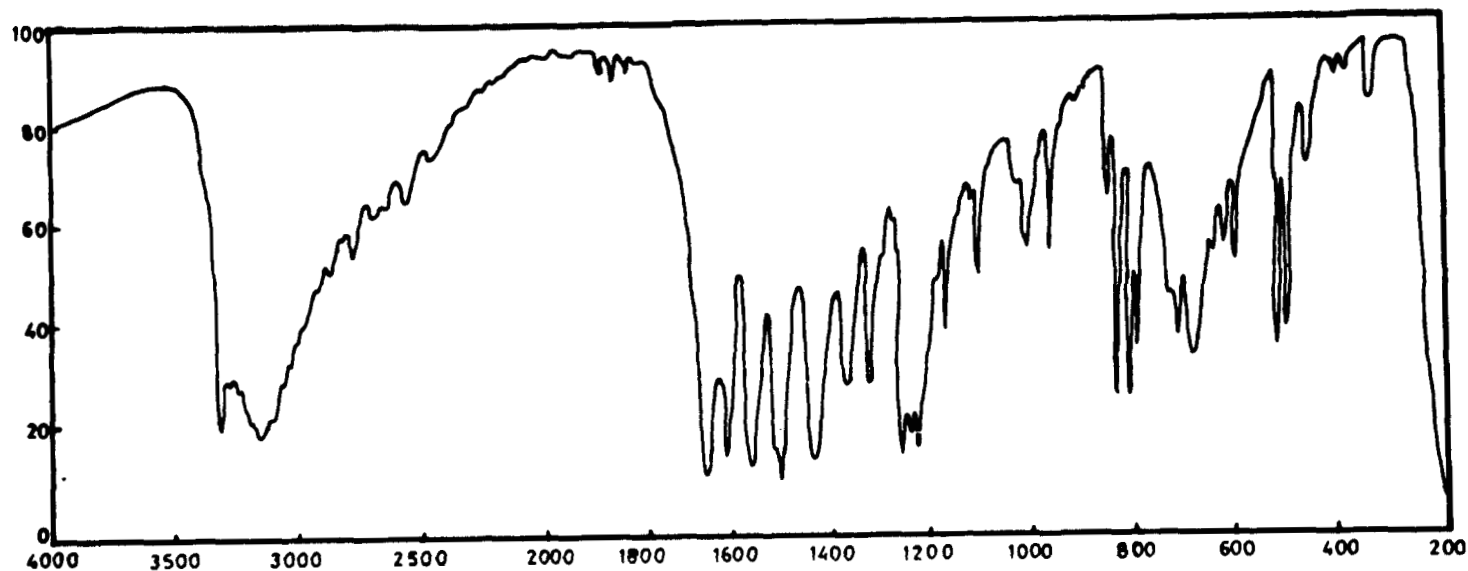


Figure 3 Infrared spectrum of acetaminophen, KBr disc.

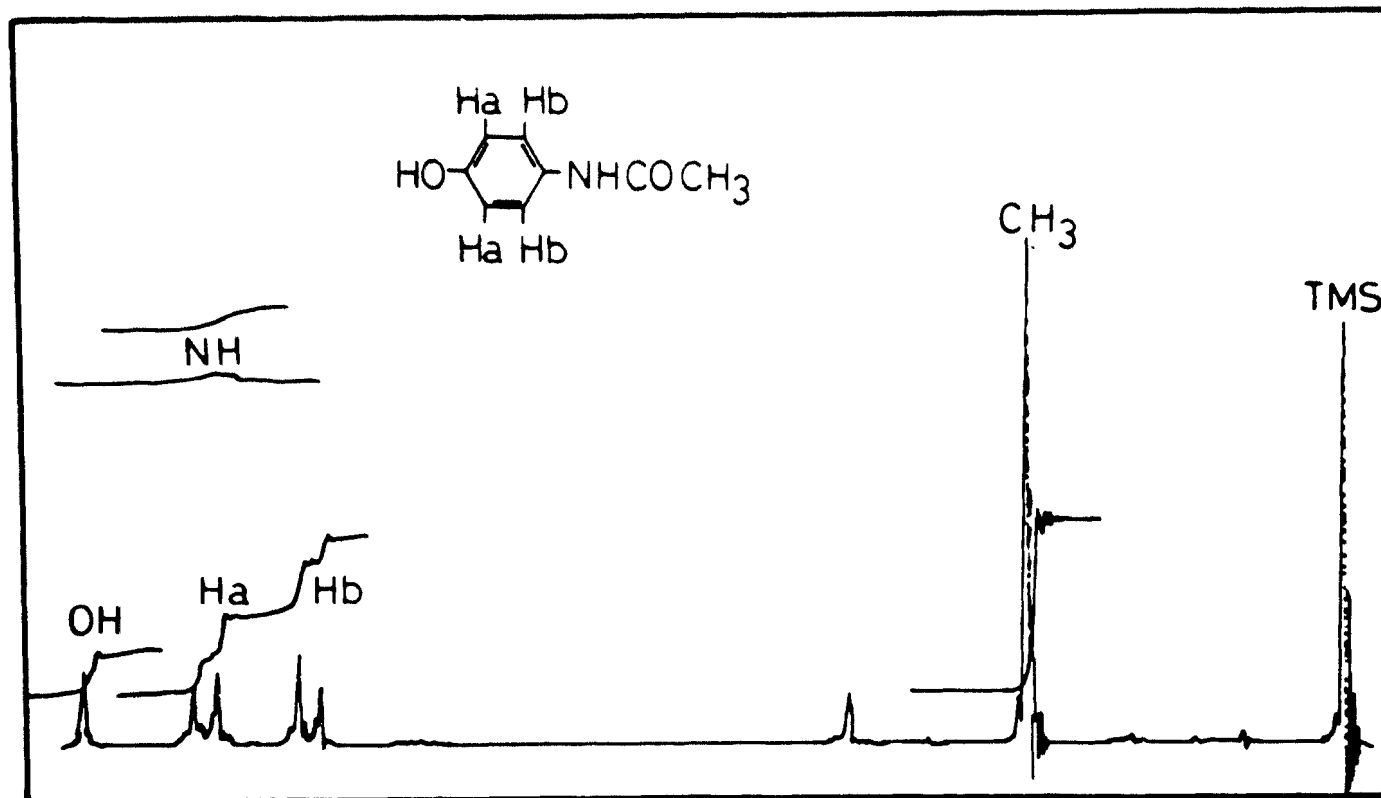


Figure 4  $^1\text{H}$  NMR spectrum of acetaminophen in acetone —  $\text{d}_6$  with TMS as internal reference.

<u>Chemical Shift (<math>\delta</math>)</u>	<u>Multiplicity</u>	<u>Proton assignment</u>
2.05	singlet	$-\text{CH}_3$
6.75	doublet	aromatic
7.44	doublet	aromatic
8.23	singlet	O-H
8.97	singlet	N-H

#### 2.3.4 $^{13}\text{C}$ Nuclear Magnetic Resonance ( $^{13}\text{C}$ NMR) Spectrum

The  $^{13}\text{C}$  NMR spectra of acetaminophen in acetone- $d_6$  using TMS as an internal reference are obtained using a Jeol FX 100 MHz spectrometer at an ambient temperature. Figure 5 and 6 represent the  $^1\text{H}$ -decoupled and off-resonance spectra respectively.

<u>Chemical Shift (ppm)</u>	<u>Multiplicity</u>	<u>Carbon assignment*</u>
32.70	quartet	$\text{C}_2$
116.37	doublet	$\text{C}_2'$
122.47	doublet	$\text{C}_3'$
132.74	singlet	$\text{C}_4'$
154.87	singlet	$\text{C}_1'$
169.38	singlet	$\text{C}_1$

\*Refer to the structure in Figure 5.

#### 2.3.5 Mass Fragmentometry

The fragmentation of acetaminophen and its metabolites has been studied by many authors. Underwood and Bowie (1) used ion cyclotron resonance to study the mechanism of ketene elimination from acetanilide and related derivatives. The elimination was shown to occur

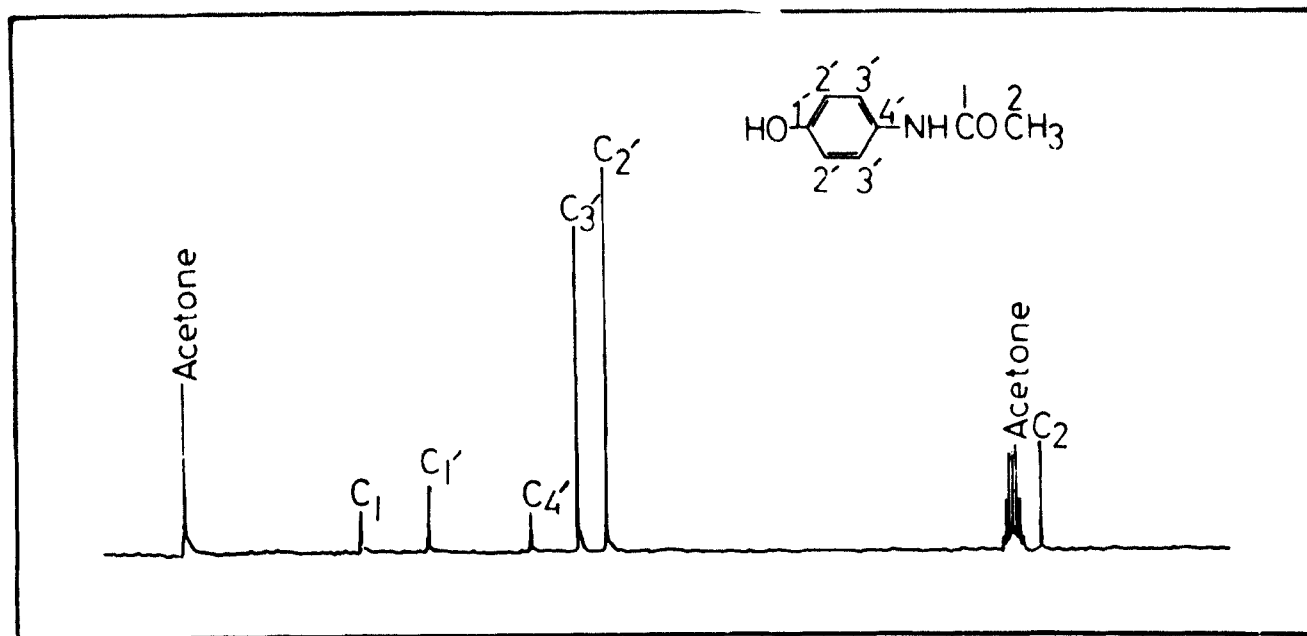


Figure 5 Noise-decoupled  $^{13}\text{C}$  NMR spectrum of acetaminophen in  $\text{acetone-d}_6$  with TMS as internal reference.

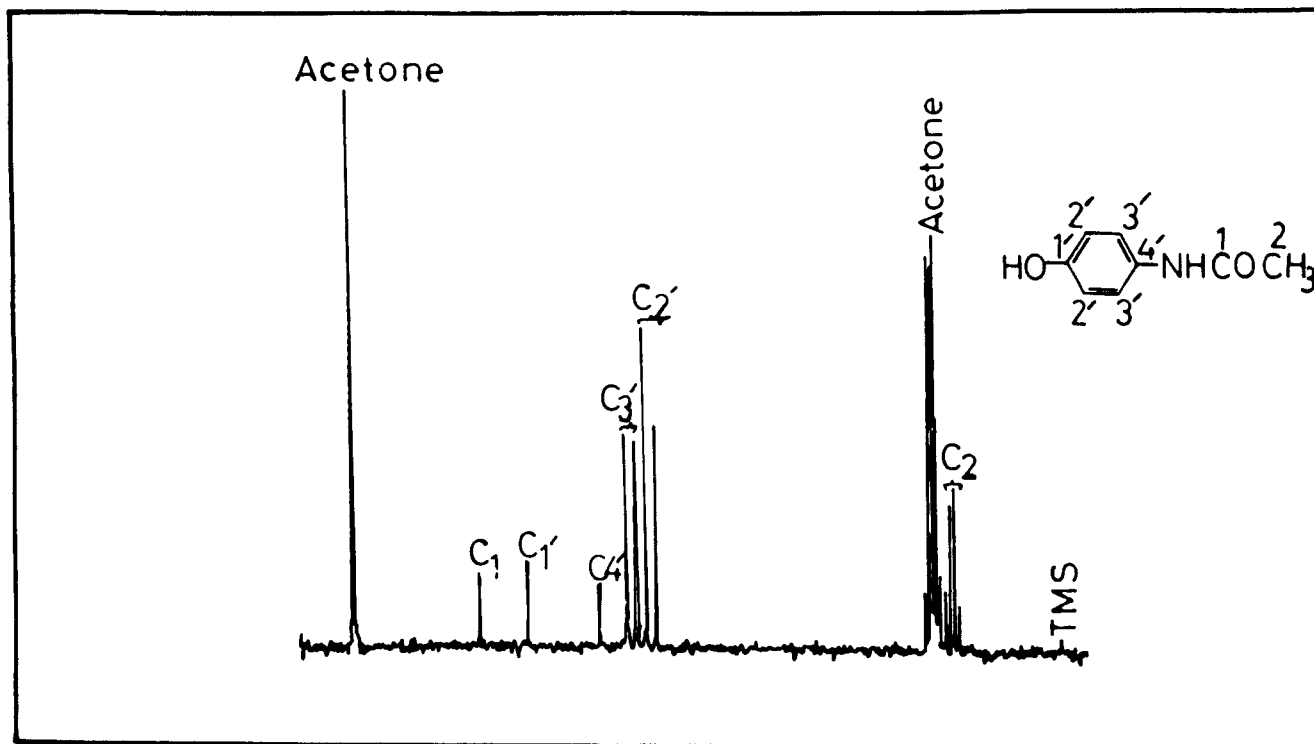
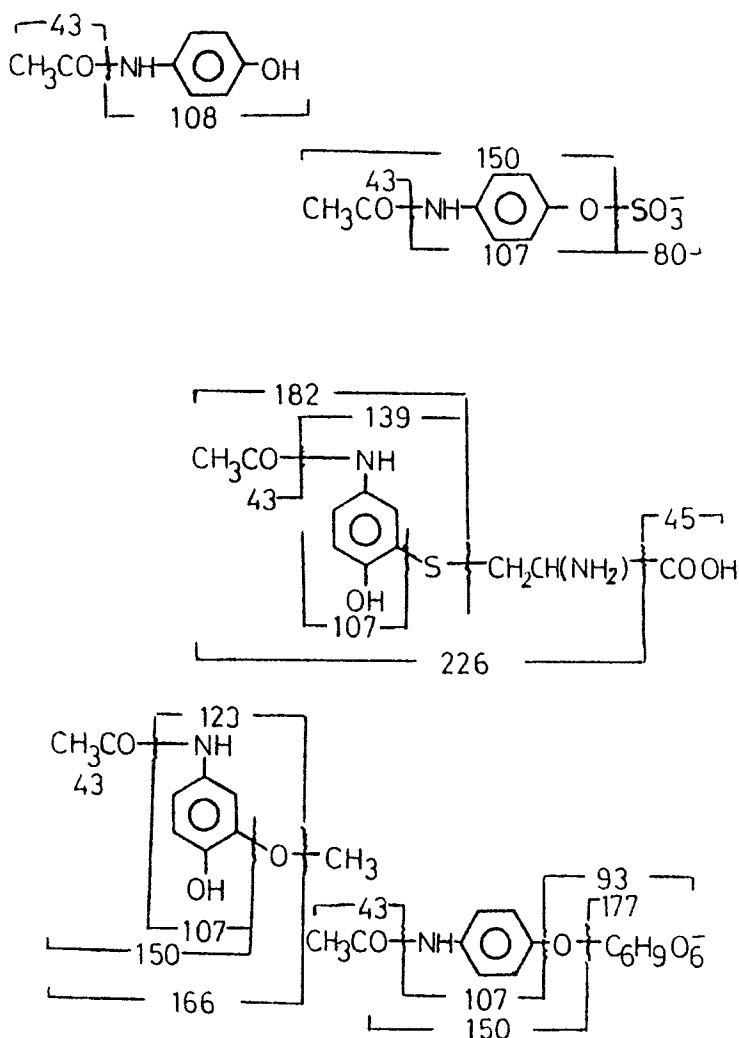


Figure 6 Off-resonance  $^{13}\text{C}$  NMR spectrum of acetaminophen in  $\text{acetone-d}_6$  with TMS as internal reference.



## SCHEME: 1

Proposed fragmentation of acetaminophen and metabolites.



through 4-membered transition states. Kinetic isotope effects ( $k_H/k_D$ ) for H-D transfer from 4- $RC_6H_4NHCOCH_2D$  ( $R = OH, MeO, H, Cl, MeCO, NO_2$ ) increase with increasing  $\sigma^+$  values of R. Kinetic energy releases occurring during ketene elimination from 4- $RC_6H_4NHAc$  radical ions increase with decreasing  $\sigma^+$ . The results are rationalized in terms of transition state geometries.

Knox and Jurand (2) carried out mass spectrometric studies on the metabolites of acetaminophen after separation from urine by HPLC. A breakdown pattern has been suggested following high-resolution mass analysis. (Scheme 1).

### 3. Pharmacokinetics

#### 3.1 Absorption and Excretion

Acetaminophen is rapidly absorbed after oral administration with peak levels obtained within 40 to 60 minutes (3, 4). Peak drug plasma levels of 640 mcg/dl in one hour for tablets have been reported. For capsules, a peak of 540 mcg/dl in 1.5 hours is reported (3). Rapid absorption of the drug was also reported (5) with mean peak plasma concentration of 21.8 mcg/dl at 23 minutes. Thomas et al (6) reported an average plasma concentration of 4.2 mg/l 6 hours after a single 324 mg dose of acetaminophen. Following oral administration of 1000 mg, serum concentration averaged 9 mg/l at 1, 2 and 3 hours (7). Serum concentrations ranging from 4.8 - 12.7 mg/l were reported by Fletterick et al (8) 30 minutes after the ingestion of 1300 mg by 4 volunteers. In a report by Prescott et al (9), 1 hour after administration of 1800 mg of the drug to 8 subjects, plasma concentrations ranged from 5.6 - 52.3 mg/l. Volume of distribution for the drug is reported to range from 0.83 - 1.36 l/kg (10).

Variations in the amount of acetaminophen absorbed occur depending on the vehicle used and the route of administration. At equivalent mg/kg doses, the oral formulation produces greater serum levels and total absorption than the propylene glycol suppository formulations which are the commercial preparations. In order to achieve a similar peak and total level, upward dosage adjustment of the suppository formulation above oral form is reasonable.

Acetaminophen has been reported to cross the placenta (11). Acetaminophen and its metabolites are excreted by the kidney. After usual doses of the drug, only 1 to 4% is reported to be excreted unchanged (4, 12, 13). It is also reported that acetaminophen does not appear to be significantly excreted unchanged in urine with values of 1.9% (14) and 3.4% with a range of 3.1-3.7% (15).

Acetaminophen half-life ranges from 2-4 hours in normal people (4, 14, 16). An average half-life of about 2 hours with a range from 0.9 - 3.25 hours is reported (3, 9, 13, 16). The drug half-life is reported unchanged in renal diseases (16). Both half-life and volume distribution of acetaminophen in children are comparable to those in adults (17).

The plasma half-life of acetaminophen is considered to be the best indicator of serious toxicity i.e. liver damage. Patients developing hepatotoxicity usually ingest at least 10-15 g of drug, exhibit a drug half-life greater than the normal 2 hours and have a plasma drug concentration of greater than 300 mg/l at 4 hours post-ingestion (18).

### 3.2 Metabolism

Following usual doses, approximately 25% of the drug is reported to be metabolized on the first pass through the liver (10). In therapeutic doses the drug is excreted largely in the urine as various conjugates: 45-55% as glucuronide conjugates, 20-30% as sulfate and 15-55% as cysteine and mercapturic acid conjugates. The unchanged drug comprises approximately 2% of the dose. The amount of unchanged acetaminophen excreted in urine after overdosage may increase to as much as 10-14% of a dose (19).

The N-deacetylation of acetaminophen in rats was found to be 6%. The major urinary metabolite were N-acetyl-p-aminophenyl sulfate and N-acetyl-p-aminophenyl glucuronide (20). The relative proportion of these metabolites varied with sex and extent of dosage.

Miners et al (21) investigated the metabolism of acetaminophen in healthy males, healthy females and healthy females receiving oral contraceptive steroids. The



drug clearance was 22% greater in males than in the control female group. This difference was entirely due to increased activity of the glucuronidation pathways in males; there was no sex-related difference in the sulfation or oxidation metabolism of the drug. Acetaminophen clearance in females using oral contraceptive steroids was 49% greater than in the control females. Glucuronidation and oxidative metabolism were both induced in oral contraceptive steroids users (by 78 and 36% respectively), but sulfation was not altered. Induction of paracetamol metabolism by oral contraceptive steroids may have clinical and toxicological consequences.

Paracetamol metabolites (Scheme 2), include paracetamol sulfate, paracetamol- $\beta$ -D-glucuronic acid, paracetamol mercapturic acid and paracetamol cysteine conjugates (22-25). 3-Hydroxylated metabolites; 3-methoxyparacetamol sulfate, 3-hydroxyparacetamol-3-sulfate and 3-methoxyparacetamol glucuronide have been reported by Andrews *et al* (24). Glycolyl and oxanilic acid derivatives formed by  $\omega$ -oxidation have also been observed (20). 3-Thiomethyl-substituted paracetamol has been observed both as the sulfone (23) and conjugated with glucuronic acid and/or sulfate (26). The double conjugate with glucuronic acid and cysteine was also postulated (25). Sulfate and glucuronic acid conjugates of p-aminophenol would be expected if deacetylation occurs.

Mudge *et al* (27) studied the covalent binding of the metabolites of acetaminophen to tissue proteins and the depletion of renal glutathione. After i.p. injection of acetaminophen to mice and rats tissue glutathione and covalent binding of tritiated metabolites to tissue protein were measured for the liver, kidney cortex and kidney papilla. Glutathione was reduced more in mice than in rats, and more in liver than in kidney without appearance of oxidized glutathione in either tissue. Covalent binding was likewise greater in mice than in rats and greater in the liver than in the kidney. The determination of covalent binding was extremely sensitive to the trace radiochemical impurities of the labeled drug. With prior administration of methylcholanthrene, the induced changes were far greater in the liver than in the kidney, suggesting that the formation of a reactive

metabolite from acetaminophen occurred in each organ by slightly different mechanisms. At doses less than those associated with demonstratable acute toxicity, the duration of covalent binding to protein was longer for renal papilla than for renal cortex or for liver. The results may be applicable to the pathogenesis of both acute and chronic nephrotoxicity.

Glutathione is required for cysteine and mercapturic acid conjugate formation; acetaminophen overdosage, causes saturation of the conjugation pathways and glutathione stores become depleted leading to the formation of a highly reactive acetaminophen metabolite, possibly an epoxide. This toxic metabolite is believed to combine irreversibly with hepatocyte constituents causing serious cell damage (24, 28) (Scheme 3).

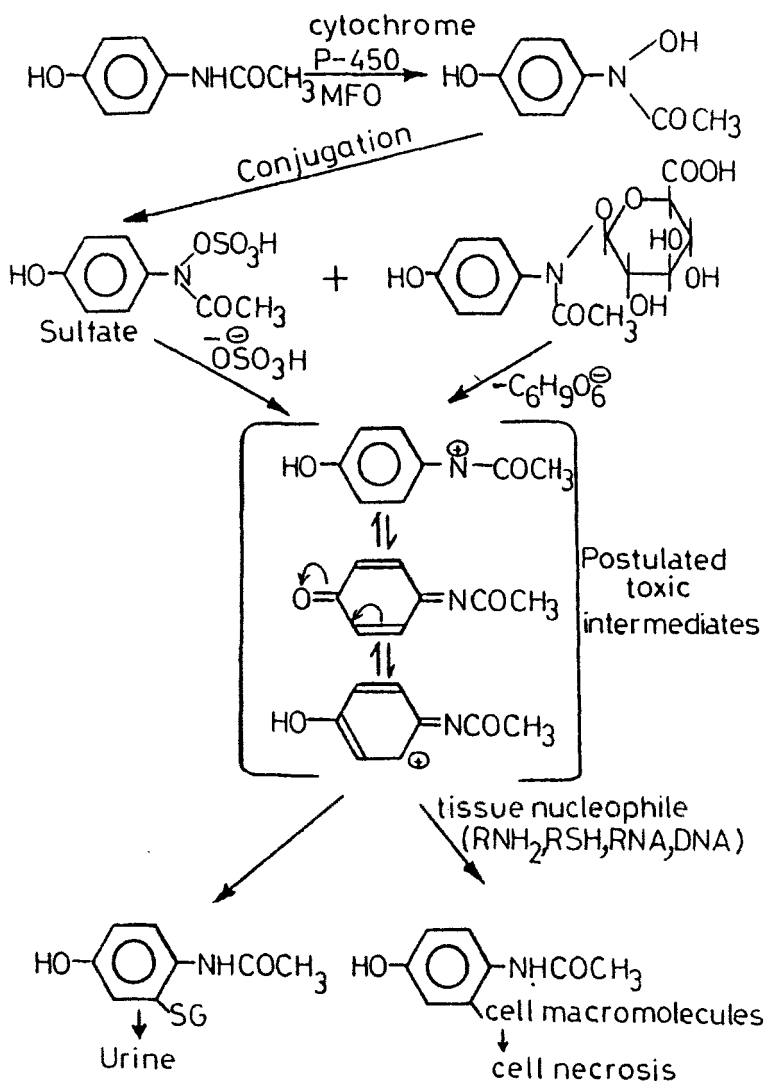
A useful antidote for acetaminophen toxicity is N-acetylcysteine. Treatment with this agent may be warranted if the half-life exceeds 4 hours (13, 29). This may be accomplished within the first 10 hours to be effective. Intravenous administration of N-acetylcysteine may have the advantage over oral administration in patients with nausea and vomiting (30). N-Acetylcysteine is believed to inactivate the toxic metabolic intermediate of acetaminophen as a glutathione analog (29).

#### 4. Methods of Analysis

Numerous methods have been reported for the analysis of acetaminophen in pharmaceuticals and in biological fluids. Colorimetric techniques have been frequently employed in clinical laboratories (31-33) but they may be subject to interference by acetaminophen metabolites, salicylate, phenacetin or uremic or icteric serum (34). Gas chromatography with (35-37) or without (38, 39) derivatization, is a more specific technique. High-pressure liquid chromatography is both sensitive and specific for clinical purposes (8, 40-44) and has the advantage of being applicable to the measurement of the conjugated metabolites of acetaminophen in plasma and urine (2, 45, 46).

## SCHEME:3

Proposed metabolic activation of acetaminophen to toxic metabolites.



#### 4.1 Titrimetric Methods

A titrimetric method for the estimation of paracetamol alone or in powdered tablets is reported (47). The powder is boiled with dilute HCl for 30 min. The solution cooled to  $< 20^{\circ}$ . A mixed indicator [(0.15% methylene blue - Tropaeolin OO(C.I. Acid Orange 5) (1:1)] and KBr are added and the solution is titrated with 0.1 M  $\text{NaNO}_2$ .

Analysis of acetaminophen and barbiturate combination has been carried out by differentiating nonaqueous titration (48). The sample is transferred to a column of Dowex 1-X8 with the aid of 50% aq. ethanol. The column is washed with 50% aq. ethanol (25 to 50 ml) and water (3 X 20 ml) and the washings discarded. The drugs are eluted with 50% ethanolic acetic acid (3 X 20 ml), and effluent evaporated to dryness, the residue dissolved in dimethylformamide and titrated potentiometrically with 0.1 N tetrabutylammonium hydroxide. Mean recovery and standard deviation for acetaminophen was  $97.8 \pm 1.6\%$ .

Mixtures containing acetaminophen, aspirin and salicylamide were assayed potentiometrically by nonaqueous titration (49). The difference in pKa values for these weak acids was sufficient to permit successful differentiation. The titrant was tetrabutylammonium hydroxide, and the titration solvent was dimethylformamide. The procedure was applied to commercial dosage forms.

#### 4.2 Electrochemical Methods

A direct method for the determination of acetaminophen in plasma by differential pulse voltammetry (50) involves mixing of the sample with 0.5 M - phosphate buffer of pH 8 and after 5 minutes, the differential pulse polarogram is recorded from + 0.2 to + 0.8 V; a carbon-paste working electrode, a silver - AgCl reference electrode and a platinum - wire auxillary electrode are used. The peak height at  $\approx 0.51$  V is a rectilinear function of paracetamol concentration from 20 to 400  $\mu\text{g/ml}$ . The coefficient of variation was  $\approx 5\%$ .



An amperometric method (51) for the determination of acetaminophen in serum involves the use of a flow-injection apparatus constructed from readily available components and incorporating a sample-introduction chamber and a thin-layer cell with a silicon-graphite grease working electrode, a platinum auxiliary electrode and a s.c.e. The method involves the extraction of acetaminophen into ethyl acetate at pH 6.0 and back-extraction of the drug into 10 mM - phosphate buffer (pH 8.5), this solution being introduced into a stream of electrode (0.1 M - phosphate buffer, pH 6) for passage through the cell. The drug was oxidized at the working electrode, and the signal produced was amplified and passed to an integrating strip-chart recorder. The detector response was rectilinear over the range 10 to 300  $\mu\text{g/ml}$  and the coefficient of variation was  $> 3\%$ .

Bezuglyi et al (52) reported an indirect polarographic determination of paracetamol in tablets, coated tablets and powders. The drug is hydrolysed with 4 M  $\text{H}_2\text{SO}_4$  at  $90^\circ$  for 30 minutes. The resulting p-aminophenol is then condensed with  $\text{HCHO}$ . The relative standard deviation was 2-5%.

#### 4.3 Spectrophotometric Methods

##### 4.3.1 Ultraviolet Methods

A UV method for the simultaneous multi-component drug determination based on the application of vidicon spectrometer is established by McDowell et al. In one paper (53) they used a detection system and an on-line small computer, to collect spectral data at repetition rate of 100 scans per second. Absorbance values at several wavelengths are processed by means of matrix equations. By this method the concentration of two or more drugs known to be present can be determined. The method is used for the determination of paracetamol in multi-component preparations in aqueous media. In a second report (54) the same authors applied multi-component least-squares calculations to the output from a visicon spectrometer and results are reported for the determination of each component in different mixtures including a mixture of caffeine, para-

cetamol, salicylic acid and salicylamide. The mean recoveries were 98% to 101%, and the relative standard errors were < 3%.

A differential spectrophotometric method involving the determination of the absorbance at 267 nm of an alkaline solution against a blank and Glenn's method of orthogonal function are described (55) for the assay of acetaminophen in tablets, syrups and suppositories. Interference from excipients in the formulations is thereby avoided.

Paracetamol is determined spectrophotometrically (56) in multi-component tablets. A quantity of the powdered tablets is dissolved in methanol, diluted with water and extracted with ethyl ether. The aqueous layer is made alkaline with 1 M-NaOH, extracted with  $\text{CHCl}_3$ . Paracetamol is determined at 264 nm in the NaOH layer. At a concentration of 7.2  $\mu\text{g/ml}$  of paracetamol the standard deviation was 0.006.

A spectrophotometric method is reported (57) for the determination of acetaminophen in the presence of its main degradation product, p-aminophenol. It involves measuring the absorbance in 0.1 N HCl between 231 nm at 10 nm intervals. By substitution in a given formula, the calculated coefficient of the quadratic orthogonal polynomial will be proportional to the drug concentration. The mean recovery of authentic samples was 100.31%.

#### 4.3.2 Colorimetric Methods

Meola (58) reported a method for the emergency determination of acetaminophen in which the serum sample or standard plus acetate buffer solution of pH 5.0 is mixed with isopropyl alcohol -  $\text{CH}_2\text{Cl}_2$  - ethyl ether (1:5:2). After centrifuging, the supernatant is mixed with carbonate buffer solution of pH 11.0 and Folin-Ciocalteu phenol reagent with which paracetamol forms a stable indophenol dye. After 25 min. the absorbance is measured at 660 nm against a blank. The absorbance is rectilinearly related to paracetamol

concentration from 5 to 120 mg/L. Recoveries are  $\approx 90\%$  and day-to-day coefficient of variations are  $\approx 8\%$ . Lack of interference from other drugs indicates good selectivity, and correlation with HPLC is good.

A method for the determination of acetaminophen concentration in human plasma is described (59) and compared with GC and HPLC methods. To the plasma sample was added aryl acylamidase followed by the color reagent, ammoniacal Cu cresol. The absorbance was read after 3 min. at 615 nm. The microbial aryl amidase used had a high degree of specificity for paracetamol and the chemical reaction of the p-aminophenol so formed, with cresol, to produce an indophenol dye is also highly specific. No drug commonly formed in proprietary paracetamol preparations interfere with the enzymic assay or give a false color reaction. Furthermore, no interferences were observed with several common, but unrelated drugs, which are sometimes taken in multiple overdose. In term of sensitivity, linearity, precision and accuracy, the enzymic assay was closely comparable to established HPLC and GC methods over the range of 0-2.5 mmol/L. Thus, the range of plasma paracetamol concentrations usually found in poisoned patients can be covered without sample dilution.

Paracetamol was also determined in plasma (60) by mixing the sample with 4N-HCl diluting with water and centrifugation. The supernatant is heated in a boiling water bath for 1 hr, extracted with ethyl ether and acidifying the extract with HCl. The acid solution is treated with 5% vanillin solution in isopropyl alcohol and the absorbance measured at 395 nm. The mean and standard deviation were  $98 \pm 5\%$ .

Miceli *et al* (32) described a rapid and simple spectrophotometric procedure for the determination of acetaminophen. Using this method the determination of acetaminophen in blood plasma gave linear results over the range 25-100  $\mu\text{g/ml}$ . Reagent required for the colorimetric procedure were 5% trichloroacetic acid, 1% orthocresol solution and 4 M -  $\text{NH}_4\text{OH}$ , and the absorbance was

measured at 615 nm. The results are comparable to those of HPLC method.

Murfin (61) reported an automated procedure for the determination of paracetamol alone or in the presence of other analgetic agents. The scheme reported involves the treatment of the drug, at room temperature, with 0.05 N HCl and NaClO to give p-benzoquinonechlorimine and the reaction of this product with 2% phenol solution to give a blue indophenol which is measured spectrophotometrically at 625 nm.

Murfin and Wragg (62) described a manual procedure for the determination of paracetamol in formulations. The procedure was developed from the automated method described previously (61). The procedure is as follows:

Shake sample (powdered tablet containing 0.35 gm of paracetamol) with 50 ml of ethanol and dilute the extract to 100 ml with water, filter, dilute a 25-ml aliquot to 250 ml and then dilute a 10-ml aliquot of this solution to 250 ml. To a mixture of 5 ml of NaClO solution (0.4% of available Cl) and enough 0.1 N - HCl to give a pH of 3.4, add 10 ml of the diluted sample solution from a pipette whose tip is just above the surface of the solution, mix and after 5 minutes add 2 ml of 0.4% NaAsO<sub>2</sub> solution, followed after a further 10 minutes by 2 ml of 6% phenol solution. Dilute to 50 ml with a borate buffer solution of pH 9.9 (1% of H<sub>3</sub>PO<sub>3</sub>, 1.2 of KCl and 0.55% of NaOH). Mix and after 30 minutes measure the extinction at 625 nm.

Daly et al (63) applied a method described previously (Analyt. Abstract 22, 329 1972) to the determination of acetaminophen in elixirs. In 3 experiments (6 determination each) the coefficient of variation were 1.16, 1.78 and 1.95%. They have also adopted the described procedure for the automated assay of individual tablets with slight modification.

Deodhar et al (64) estimated paracetamol, in pharmaceutical formulations, colorimetrically. The method involves converting either drug into

aceto-hydroxamic acid which forms a colored complex with  $\text{Fe}^{3+}$ . To the sample of the standard solution (5 ml) add 4 N-NaOH (1 ml), after 5 minutes add 2 M-hydroxylammonium chloride (1 ml), heat the solution on a boiling-water bath for 10 minutes, cool to room temperature and add methanol (5 ml). Add water (15 ml), 5 N-HCl (1 ml), and 6%  $\text{FeCl}_3$  solution in 0.1 N - HCl (1 ml). Dilute the mixture to 25 ml with  $\text{H}_2\text{O}$ . Extract with ether (50 ml) and measure the extinction of the aqueous layer at 520 nm against a reagent blank.

A selective colorimetric method for the determination of paracetamol by means of an indophenol reaction has been reported (65). The method depends on the spontaneous oxidation of the alkaline mixtures of 4-aminophenol and phenol with oxygen after hydrolysis of paracetamol. The extinction is measured at 625 nm.

Iovchev et al (66) applied "ferri-hydroxamic" complex formation to the photocolorimetric determination of paracetamol. Mathematical experiments were conducted for establishing optimum conditions for hydroxyl-aminolysis of paracetamol during its quantitative determination as hydroxamate. A solution of the drug in 95% ethanol was heated at  $72^\circ$  for 55 minutes, with 4 M-hydroxylammonium chloride in 4 M-NaOH. The mixture was acidified to pH 2 with 4 M HCl, 0.3 M- $(\text{NH}_4)_2 \text{SO}_4\text{-Fe}_2 (\text{SO}_4)_3$  was added, and after dilution with isopropyl alcohol- $\text{H}_2\text{O}$  (3:1), the absorbance was measured at 520 nm.

Paracetamol is determined in pharmaceuticals, colorimetrically, by the procedure described by Domagalina and Zareba (67). The drug sample was hydrolysed by boiling with 10% HCl; an aliquot of the hydrolysate was diluted with water and mixed with 0.1 M-  $\text{K}_3\text{Fe}(\text{CN})_6$ , 0.1 M- phenol and aqueous 5% NaOH, and, after 45 minutes, the absorbance of the indophenol formed was measured at 627 nm.

Walberg (68) determined acetaminophen in serum. The procedure is a modification of the colorimetric method of Glynn and Kendal (69). Changes

in the concentration and amounts of reagents have been made whereby the absorbance at 430 nm have been increased from 0.16 (with 12.5 ml of final solution) to 0.44 (4.5 ml of solution) for 10 mg/dl of paracetamol. The method was not recommended for absolute determination but is useful for identification of paracetamol and for establishing whether or not an overdose have been ingested.

Acetaminophen alone or in pharmaceutical preparation has been determined colorimetrically (70). The authors have analysed the drug using a modification of a previously described colorimetric procedure involving reaction with  $\text{HNO}_2$ . The absorbance at 430 nm was measured for the  $\text{HNO}_2$  - reaction products in alkaline and in acidic media, the difference between the two readings being taken as a measure for the acetaminophen content. The method is not liable to interference by drugs such as salicylamide and phenacetin.

Rana and Chauhan (71) reported a colorimetric estimation of paracetamol in pharmaceutical formulations. The formulation containing the drug was treated with diazotized 2-nitroaniline, and the absorbance of the resulting red color was measured at 520 nm. The absorbance was rectilinearly related to the paracetamol concentration over the range 0.5 to 1.5 mg/ml. The method was applied to elixires, tablets, a syrup and injection. No interference from other ingredient.

Hassan et al (72) reported a colorimetric determination of certain phenol derivatives in pharmaceutical preparations. The colored azo-dye formed from paracetamol with either sulphanilic acid or 4-nitroaniline was used as basis for determining the phenols in formulations. Measurement was made at 480 or 425 nm respectively.

Gupta et al (73) described a colorimetric procedure for the emergency determination of acetaminophen in plasma. The drug is extracted into ethyl acetate at physiological pH to eliminate salicylate, amino acids and other polar compounds.

The extract is treated with Fuller's earth to remove bilirubin and with anhydrous sodium sulfate to remove traces of aqueous droplets containing proteins or uric acid. The extract is back-extracted into carbonate and simultaneously treated with Folin-Ciocalteu reagent to produce a stable color complex. The absorbance is determined at 660 nm. Any compound which is present in the final carbonate layer and is readily oxidizable can produce false positive results for acetaminophen.

Quantitative determination of acetaminophen in serum using a colorimetric method has been published (74). The procedure involves preparing a protein-free filtrate by the addition of  $\text{Cl}_3\text{CCOOH}$ , hydrolysing the drug in the filtrate to p-aminophenol, and subsequent reaction of the p-aminophenol with phenol and ammonium hydroxide to form an indophenol blue chromogen. The absorbance at 620 nm of this chromogen follows Beer's law up to an acetaminophen concentration of 60 mg/L.

#### 4.3.3 Fluorimetric Methods

Kaito *et al* (75) reported a procedure for the determination of paracetamol. A blue-violet fluorescence, with an absorption maximum at 303 nm in ethanol, developed when paracetamol was oxidized with  $\text{K}_3\text{Fe}(\text{CN})_6$  in slightly alkaline solution, and its intensity is increased by the addition of dimethylformamide.

Kaito and Sagara (76) described a fluorimetric method for the analysis of p-aminophenol and its application to the determination of paracetamol. The method was based on the hydrolysis of paracetamol with HCl to p-aminophenol and reaction with benzylamine at 75° in alkaline solution to give a fluorescent substance.

Dolegeal-Vendrelly and Guernet (77) determined paracetamol in plasma by spectrofluorimetry. The biological fluid (2 ml) is shaken with 20 ml of ethyl ether for five minutes and ether phase is evaporated. To the residue is added 3N- $\text{H}_2\text{SO}_4$

(5 ml) and the mixture is heated for 1 hour on a boiling-water bath. After cooling to room temperature, the solution is filtered (if necessary) and the paracetamol is determined by spectrofluorimetry of its hydrolysis product, 4-aminophenol, with excitation at 280 nm and emission measurement at 370 nm. The calibration graph is rectilinear for the equivalent of 0.5 to 50 µg/ml of paracetamol in the final solution.

#### 4.3.4 Nuclear Magnetic Resonance

High-resolution proton nuclear magnetic resonance spectroscopy was used for rapid multi-component analysis of urine (78). The metabolism of simple nonendogenous compounds such as acetaminophen was monitored in 400 and 500-Hz  $^1\text{H}$  NMR spectra of intact human urine. The pH-dependencies of the NMR chemical shifts of some urine components were reported. High-resolution  $^1\text{H}$  NMR spectroscopy provides a fast, simple method for fingerprint identification of urinary compounds. Numerous low-Mr metabolites, e.g. creatinine, glucose etc. were also identified - more details have been provided regarding the presence of these compounds and its relation to the specific conditions of the donors; humans in different physiological states (resting, fasting or post-exercise) and pathological conditions (e.g. diabetes mellitus.).

#### 4.3.5 Mass Spectrometric Methods

Qualitative analysis of sedatives and antipyretics including acetaminophen has been carried out by Kanamori (79) using chemical-ionization mass spectroscopy. Applications of the method for the analysis of common preparations are also presented.

A simple and rapid analytical method using direct-inlet chemical-ionization mass spectrometry (80) was developed for the simultaneous determination of acetaminophen, o-ethoxybenzamide, phenacetin, caffeine, diphenhydramine, dextromethorphan and chlorpheniramine in anticold drugs. The drugs were detected as quasimol. ions by



CI-MS and quantitated using accumulation program. The recoveries were 97.9-100.8% and the standard deviations were 0.1-3.2%.

#### 4.4 Chromatographic Methods

##### 4.4.1 Thin-Layer Chromatography (TLC)

Symonds and Dedicoat (81) reported the identification of paracetamol in certain analgesic preparations using TLC. The ingredient was extracted with chloroform and separated by chromatography on silica gel sheets (Kodak 6061), activated to 105°C for 30 minutes, with chloroform - isopropanol (19:5) as developing solvent. The chromatogram was detected by spraying with molybdophosphoric acid solution ( $R_f = 0.40$ ).

A method for quantitative evaluation of thin-layer chromatograms has been reported by Falk and Krummen (82). A device (Camag Eluchrom) is described for extracting up to six spots simultaneously, in circular elution chambers, with small volume of methanol (2.5 ml) under pressure from the t.l.c. plate into a cell, where the extinction or fluorescence of the extract is measured. Recoveries from the plates were 96.3 to 101.4%. Down to 5 µg of paracetamol could be determined.

Ebel and Herold (83) reported the evaluation of thin-layer chromatograms with an internal standard (Two-Wavelength measuring technique with a zig-zag scan). Paracetamol was used as an internal standard in a mixture of caffeine, paracetamol and aspirin.

Paracetamol and other analgesics have been determined by thin-layer chromatography (84). The quantitative evaluation of the t.l. chromatograms for multi-component analyses is based on calibration graphs or use of internal standard.

##### 4.4.2 Gas Chromatography (GC)

Aaroe et al (85) described a GC method for the determination of paracetamol and analgesic drugs

in multi-component tablets. The method was devised for analysing tablets containing paracetamol along with other components.

The quantitative determination of acetaminophen as a main metabolite of phenacetin in blood plasma of animals has been reported by Kyo and Niwa (86). The substances were extracted from acidified plasma at pH 2.5 with ether-chloroform (1:1) containing phenanthrene as internal standard substance. The extract was concentrated and treated with N-methyl-N-trimethylsilyltri-fluoroacetamide. The silylated sample was analyzed by gas chromatography as follows:

3 mm X 2 m glass column, packed with 3% OV-17 on Shimalite: 5% SE-30 on Shimalite (10:29). Temperature was at first maintained at 130° isothermally, then programmed to increase from 130 to 170° at 1°/minute rate. The method was employed to determine the plasma level of the drug after oral administration to rats and rabbits.

Becherucci et al (87) determined paracetamol in plasma by gas chromatography. To plasma (1 ml) plus phenobarbitone (40 µg) as internal standard was added aqueous 15% trichloroacetic acid (50 µl) and the solution was agitated on a vortex-type, mixed for 5 seconds before ethyl acetate (4 ml) was added. Mixing was continued for 30 seconds. After centrifugation, the separated organic phase was evaporated to dryness at 40° under nitrogen. The residue was dissolved in 0.2 M - trimethylanilinium hydroxide (50 µl) and an aliquot (2 µl) was injected on to a column (1.83 m X 4 mm) packed with 3% OV-17 on Gas-Chrome Q (100 to 120 mesh) and operated at 190° with nitrogen as carrier gas 190° (40 ml/min) and f.i.d. The minimum detectable concentration was 0.5 µg/ml and the analyses took about 20 minutes.

#### 4.4.3 Liquid Chromatography (LC)

Baum and Cantwell (88) reported the use of liquid chromatography for the analysis of paracetamol

and other analgesics on Amberlite XAD-7. The nonionic polyacrylate resin is used as a stationary phase for the separation of mixtures of analgesics. Binary and ternary solvent mixtures prepared from chloroform, ether, ethanol and hexane are used as the mobile phase. From plots of log adjusted retention volume of each compound vs. solvent composition, the optimum solvent mixture is chosen to achieve a given separation. A mixture of six compound including paracetamol is base-line resolved on a 30 cm X 0.28 cm column.

Fletcher et al (8) determined paracetamol in serum using liquid chromatography. The drug was extracted from serum (1 ml), to which 1 ml of sodium acetate buffer (pH 4.8) had been added, with 10 ml of ethyl acetate, the solvent was evaporated, and the residue was dissolved in 100  $\mu$ l of ethanol; the 2'- and 3'-hydroxy isomers of paracetamol were added as internal standards. Separation was effected on a column (25 cm X 2.1 mm) of ZORBAXSIL, silica (5 to 6  $\mu$ m), with H<sub>2</sub>O - saturated chloroform-heptane-ethyl alcohol-anhydrous acetic acid (540:360:100:0.72) as mobile phase and detection at 254 nm. The calibration graph was rectilinear for 0.5 to 300 mg/l of paracetamol, and the recovery was 97%. Many commonly administered drugs did not interfere with the assay.

#### 4.4.4 Gas-Liquid Chromatography (GLC)

Street (89) reported the estimation and identification of paracetamol in blood in the presence of barbiturates. The interference by barbiturates was avoided by benzylation of the paracetamol. 4-Butyramidophenol was used as the internal standard. After the described extraction, the residue was dissolved in acetone (0.2 ml), and 30  $\mu$ l of this solution was submitted to g.l.c. on a stainless-steel column (6 ft X 0.125 inch o.d.) packed with Chromosorb G (100 to 120 mesh) coated with SE-52 and operated at 220° with nitrogen as carrier gas (90 ml/min) and flame ionisation detection. Paracetamol was also identified by treating

another portion of the acetone solution with NO-bis (trimethylsilyl)acetamide and subjecting to g.l.c. The method was suitable for the range 2 to 40  $\mu\text{g/ml}$ ; at the level of 10  $\mu\text{g/ml}$ , the coefficient of variation was 3%.

A simple and rapid procedure was devised by Sharman (90) for determining paracetamol in extract of plasma or aqueous solution by g.l.c. on a glass column (2 mm X 3 mm) packed with 30% of OV-225 on acid-washed and silanized Chromosorb W, with thermionic detection.

Windorfer and Roettger (91) described a g.l.c. procedure for the micro-determination of paracetamol and other antipyretic agents. The method was used to study blood levels of active principles and metabolites in patients under treatment with more than one antipyretic. Glass column (4 m X 0.2 mm) packed with 4% of silicone oil DC 200 on Gas-Chrom Q were used. Details are given for the determination of the drug in 0.5 ml of serum. The limit of detection was 1  $\mu\text{g}$  per ml for paracetamol.

Thoma *et al* (36) reported an improved gas chromatographic assay of paracetamol. It is a modification of a method reported earlier by Evans and Harbison (92). Serum (200  $\mu\text{l}$ ) plus 2-acetamidophenol (internal standard) is mixed mechanically with  $(\text{NH}_4)_2\text{SO}_4$  and the mixture is shaken with dichloromethane. The organic layer is separated and a portion is treated with acetic anhydride and pyridine. The mixture is evaporated under N at 40° to 50°, and a solution of the residue in dichloromethane is analysed by g.l.c. at 180° on a column (90 cm X 2 mm) packed with 3% of SP-2250-DA on Supelcoport (100 to 120 mesh), with nitrogen as carrier gas (40 ml/min) and flame ionisation detection. Retention times (min) are 3.2 for the derivative of paracetamol and 1.2 for that of the internal standard. The detection limit is 5 mg/l in the sample, and the calibration graph is rectilinear for 50 to 500 mg/l. Of twenty other drugs likely to be present, only meprobamate gives a peak near that for the derivative of paracetamol.

Street et al (93) reported the preparation of low-activity packed columns and applied them to the toxicological analysis of underivatized polar drugs in low-nanogram range. Chromosorb G-NAW was left in contact with pyridine-benzoyl chloride (3:2) for 48 hours, then washed with acetone until free from pyridine. This acylated support material was suspended in 10% SE-52 solution in toluene for 48 hours, filtered off under nitrogen, dried and heated at 410° for 1 hour. A pyrex-glass column (6 ft X 0.125 in o.d.) was filled with pyridine-benzoyl chloride (3:2), set aside for three days, emptied, rinsed with toluene and dried at 100°. The column was then either (a) twice filled with 5% of SE-52 in chloroform and emptied, dried at 100°, then dried at 350° under nitrogen for 10 hours, before filling with support - or (b) filled with the support heated at 400° under nitrogen for 1 hour, emptied, refilled with the support and heated at 350° under nitrogen in the gas chromatograph until a stable base-line was obtained. In this way columns with reduced activity were obtained, enabling detection limits to be extended to 1 ng for unmodified polar compounds. e.g. paracetamol. This results in an "improvement" in capability about 1000-fold compared with a commercial OV-1 column.

The effect of paracetamol on amylobarbitone hydroxylation in man was studied using gas chromatographic method in the simultaneous estimation of underivatized paracetamol and barbiturates (94). Paracetamol and barbiturates were determined in plasma by extraction at pH 5.5 with ethylacetate and g.l.c. on a column containing 0.33% of CDMS on Chromosorb A (HP) which was temperature-programmed, after 4 minutes, at 170° to 225° at 10°/min; alkaline-flame ionisation detection was used. No endogenous compounds interfered and the coefficient of variation between assays were 5-6% (for paracetamol at 10 µg/ml).

#### 4.4.5 High-Pressure Liquid Chromatography (HPLC)

HPLC has been extensively used for the determination of acetaminophen in pharmaceutical

formulations and in biological fluids.

Horvitz and Jatlow (95) reported a method for the determination of acetaminophen concentration in serum by HPLC. Serum plus solid NaCl is extracted with a solution of 4-propionamidophenol (internal standard) in ethyl ether. The ether phase is evaporated to dryness at room temperature, and a solution of the residue in methanol is applied to a column (25 cm X 4.6 mm) of Partisil - 10 ODS operated at 40°, with a 5% (v/v) solution of acetonitrile in 0.1 M potassium phosphate buffer of pH 2.7 as mobile phase (1 ml/min) and detection at 250 nm. Retention times are 5 min. for paracetamol and 6.2 min. for the standard, and the calibration graph (peak-height ratio vs concentration) is rectilinear for 10 to 100 mg/L of the drug. A slightly modified procedure yields a calibration graph rectilinear in the range of 1 to 20 mg/L. Commonly used drugs do not interfere; hydrochlorothiazide and sulfadiazine could interfere, but their presence in conjunction with paracetamol is considered to be unlikely.

A liquid chromatographic method for the determination of muscle-relaxant analgesic mixtures using reverse-phase and ion-pair techniques is reported by Stewart et al (96). Retention times were measured on columns (30 cm X 4 mm) of  $\mu$  Bondapak C<sub>18</sub> by HPLC with different solvent systems. Paracetamol was separated with aq. 50% methanol as the mobile phase. Recovery was  $\approx$  103%.

Trace determination of acetaminophen in serum was carried out by Miner and Kissinger (97) as follows:

The sample is saturated with NaCl and extracted (at pH 7) with CH<sub>2</sub>Cl<sub>2</sub> - isopropyl alcohol - ethyl ether (5:1:2). The extract is evaporated to dryness under N at 40°, and a solution of the residue in the mobile is submitted to HPLC on a column (15 cm X 4.6 mm) of  $\mu$  Bondapak with water-1 M-ammonium acetate-methanol-1 M-acetic acid (390:34:70:9) as the mobile phase and a carbon-paste electrochemical detector (+ 0.65 V vs. the

s.c.e.). N-(4-Hydroxyphenyl) propionamide is used as internal standard. The peak height is a rectilinear of function of the concentration of paracetamol from 0.02 to 20  $\mu\text{g/ml}$ . The coefficient of variation was 3% at 2  $\mu\text{g/ml}$  and there was no interference from other drugs tested.

A quantitative method for the estimation of the glutathione, cysteine and N-acetyl cysteine conjugates of acetaminophen obtained from microsomal incubations has been developed (98) using HPLC. A reverse phase  $\text{C}_{18}$   $\mu$  = Bondapak column was used and eluted with 12.5% methanol: 1% glacial acetic acid: 86.5% water or 10% methanol: 1% glacial acetic acid: 89% water containing 0.005 M 1-heptane sulfuric acid or 8% acetonitrile: 92% water. As little as 5 ng of the thiol conjugates of [ring- $^{14}\text{C}$ ] acetaminophen can be measured with good precision from microsomal incubations with minimal sample preparation. HPLC offers distinct advantages over the standard separation techniques of thin-layer, paper or gel filtration chromatography for the separation and quantitation of the polar thiol metabolites of acetaminophen.

An improved HPLC method for the separation of urinary paracetamol metabolites using radially compressed columns is recently published by Harts et al (99). Enhanced resolution and decreased analysis time were major advances. Various modifications of existing methods were made to counter the effect of the different  $\text{C}_{18}$  surface. Thus, in ion-suppression HPLC the addition of  $\text{Et}_3\text{N}$  at pH 3 (phosphate buffer) was necessary to block residual OH sites, while in ion-pair HPLC a higher  $\text{Bu}_4\text{NOH}$  concentration of 0.01 M at pH 5 was used to enhance selectivity. The methods were successfully applied to the study of the metabolites of paracetamol, paracetamol glutathione conjugate and 3-thiomethylparacetamol in rats. 3-Thiomethylparacetamol sulfoxide, its glucuronide conjugate and its sulfate conjugate were metabolites of both 3-thiomethylparacetamol and paracetamol. 3-Thiomethylparacetamol sulfate was unresolved from paracetamol sulfate and 3-methoxyparacetamol sulfate in ion-pair HPLC.

This raises a previously unrecognized problem, in which the peak normally attributed to paracetamol sulfate contains metabolites arising from an oxidative metabolic pathway. Elevated levels of 3-methoxyparacetamol conjugates were found in human overdose urine and to some extent in analgesic nephropathy.

Other HPLC systems for the separation and determination of the drug has also been reported (100-103). Furthermore, a review, on the use of HPLC for the examination of analgesics which includes experimental details of absorption chromatography of the drug, have been published (104).

Table 1, shows the chromatographic condition for other reported HPLC procedures.

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Table 1. HPLC methods for the analysis of acetaminophen.

Stationary Phase	Mobile Phase	Flow rate	Detection	Remarks	Ref.
60 cm X 2.1 mm Corasil II	Acetic acid, chloro- form and dichloro- methane (4:21:25)	1 ml/min	U.V. at 254 nm	An automated system	105
100 cm X 0.22 cm Bio- Rad A-27 resin (10 to 12 $\mu$ m).	0.015 M - sodium acetate - acetic acid buffer at pH 4.4.	8.5 ml/h	U.V. at 254 or 280 nm and by fluori- metry.	*The column is equilibrated with 0.015 M - sodium acetate-acetic acid buffer of pH 4.4. *The column is maintained at room temperature for 80 minutes and at 70° for the rest of the run	106
30 cm X 2 mm Pellidon	40 mM - phosphate buffer at pH 7.4	0.35 ml/min	Amperometric detector	*The method is used for the determina- tion of the drug in tablet, liquid dosage forms as well as in serum and urine	107

Table 1. (Continued)

Stationary Phase	Mobile Phase	Flow rate	Detection	Remarks	Ref.
12.5 cm X 5 or 7 mm Octadecyl silica (6 $\mu$ m mean particle diameter) or octadecyl silica silinized).	Water: methanol: formic acid (85:15: 0.15).		U.V. at 254 nm	*Used for the separation of the drug and its main metabolites. *Better results were obtained using silinized column.	2
Two columns:- 5 cm X 0.055 inch 50 cm X 0.055 inch connected in a series. Aminex A-5 (13 $\pm$ 0.2 $\mu$ m)	0.45 M - $\text{NH}_4\text{H}_2\text{PO}_4$ buffer (pH 3)	0.2 ml/min.	U.V. at 254 nm	*A micro scale method. *The N-butyryl analoge of paracetamol was used as internal standard.	108
25 cm X 2.1 mm Micropak CN	Hexane-dichloro- methane-methanol- acetic acid of vary- ing methanol content	-	UV at 254 nm	*Used for identification and quantitation of the drug as an impurity from benorylate (Salipran).	109
Li Chrosorb SI-60 (5 $\mu$ m)				Detection limit for paracetamol was 10ppm	110

Table 1. (Continued)

Stationary Phase	Mobile Phase	Flow rate	Detection	Remarks	Ref.
30 cm X 3.99 mm $\mu$ Bondapak $C_{18}$	3.5% acetonitrile solution in sodium acetate buffer solu- tion (pH 4).	2 ml/min.	U.V. 254 nm	*Used for the deter- mination of the drug in plasma. *N-(2-hydroxyphenyl) acetamide was used as internal standard	111
10 cm X 8 mm Radial-PAK $C_{18}$ cartridge	Acetonitrile-metha- nol-water (3:3:44).	1.8-2.2 ml/min	U.V. 254 nm	*Used for the deter- mination of the drug in plasma. *N-(2-hydroxyphenyl) acetamide was standard.	111
30 cm X 3.9 mm $\mu$ Bondapak $C_{18}$ (10 $\mu$ m)	Methanol-aq. acetic acid (1 ml/l) (3:7)	2.5 ml/min.	U.V. at 250 nm	Rapid method for the analysis of the drug in blood and post- mortem viscera.	112
30 cm X 3.9 mm $\mu$ Bondapak $C_{18}$ (10 $\mu$ m)	1% acetic acid-metha- nol-ethylacetate (900:150:1).			A reversal phase chromatography of urinary metabolites of paracetamol using suppression and ion- pairing.	113

Table 1. (Continued)

Stationary Phase	Mobile Phase	Flow rate	Detection	Remarks	Ref.
25 cm X 4.6 mm Nucleosil 5 C <sub>18</sub>	Methanol-phosphate buffer solution of pH 3 (4:5).	1 ml/min	U V 240 nm	*Salicylamide was used as an internal standard. *Detection limit was 1 mg/l.	114
30 cm X 4 mm, reverse phase, high efficiency C <sub>18</sub> column fitted with a precolumn packed with C <sub>18</sub> Corasil 37- 50 µm.	Methanol-water (15:85)	1 ml/min	U. V 240 nm	*Rapid method for the quantitative determination of the drug in plasma.	115

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# HALOTHANE

Chester E. Orzech and  
Frederick Q. Gemmill, Jr.

*Ayerst Laboratories, Inc.  
Rouses Point, New York*

1. Description	598
2. Physical Properties	598
2.1 Infrared Spectra	598
2.2 Nuclear Magnetic Resonance Spectra	598
2.3 Ultraviolet Spectra	598
2.5 Optical Rotation	599
2.6 Vapor Pressure and Boiling Point	599
2.7 Density	599
2.8 Refractive Index	600
2.9 Solubility	600
2.10 Hydrates/Clathrates	601
2.11 Miscellaneous	601
3. Synthesis and Purification	601
3.1 Synthesis	601
3.2 Impurities	602
3.3 Purification	602
4. Stability and Degradation	604
4.1 Stability	604
4.2 Inflammability	604
4.3 Degradation	604
5. Drug Metabolic Products	604
6. Methods of Analysis	605
6.1 Analysis of Halothane in Mixtures	605
7. Determination in Body Fluids and Tissues	607
7.1 Gas Chromatographic Methods	607
7.2 Absorptiometric Methods	607
7.4 Electrochemical	607
7.5 Liquid Scintillation	608
References	608

The following supplement contains new information on the analytical chemistry of halothane obtained from an updated literature survey to September 1983. The numbering system is the same as that used in the original profile (Volume 1, pp. 119-147).

## 1. Description

Halothane is a polyhalogenated hydrocarbon used as an inhalation anesthetic.

1.3 The CA registry number for halothane is 151-67-7.

## 2. Physical Properties

### 2.1 Infrared Spectra

Nagyrevi and Sandorfy (1) made a study of the infrared spectrum of halothane at low temperature in 2-methyl-tetrahydrofuran and showed that halothane shifts H-bond equilibrium involving water in favor of less H-bonding. Hornischer and Moser (2) measured the vapor state Raman intensities with Hg-arc excitation and photoelectric detection. Schwartzman (3) published a spectrum for the vapor infrared spectrophotometric identification of halothane. The hydrogen bonding properties of halothane in several systems have been investigated by infrared spectrophotometry (4-6).

### 2.2 Nuclear Magnetic Resonance Spectra

The proton spectrum chemical shifts and coupling constants were obtained by Keda (7). Brown and Chaloner (8) obtained proton spectra as a function of solvent to show the effect of H-bonding. Addition of N-methylpyrrolidone caused a substantial downfield shift in the proton spectrum due to formation of a 1:1 H-bonded complex. The fluorine-19 spectrum of halothane was obtained Keda (7) and the fluorine-19 chemical shifts measured as a function of solvent (9-11).

### 2.3 Ultraviolet Spectra

Barrett and Nunn (12) and Dumas et al (13) have published the far ultraviolet spectrum of halothane. Dumas et al (13) also have published the photoelectron spectrum. The maximum is at 204 nm in nitrogen gas while in water the maximum is at 200 nm (12). A 1% mixture of halothane in nitrogen gave an absorbance of 0.132 at 204 nm in a 10 mm cell (12).

## 2.5 Optical Rotation

Optically active halothane was prepared by Edamora et al (14). An 80/20 mixture of d- and l-isomers had a rotation of  $+1.0^{\circ}$  at  $25^{\circ}\text{C}$  and a 27/73 mixture a rotation of  $-0.64^{\circ}$  using the D line of sodium.

## 2.6 Vapor Pressure and Boiling Point

Rodgers and Hill (15) reported the use of a hand-held programmable calculator to obtain Antoine equation coefficients from experimental data for halothane:

$$\log_{10} P = A - \frac{B}{t+C}$$

Where

P = pressure

A = 5.89184 kPa, or  
6.76799 mmHg, or  
3.88718 atm.

B = 1043.697

C = 218.262

t = temperature,  $^{\circ}\text{C}$

Gmehling et al (16) also obtained Antoine equation coefficients (A=6.53534 mmHg, B = 915.201, C = 200.229). They also reported vapor pressure data as follows:

<u>t, <math>^{\circ}\text{C}</math></u>	<u>P(mmHg)</u>	<u>t, <math>^{\circ}\text{C}</math></u>	<u>P(mmHg)</u>
25.60	303.70	40.88	548.59
28.95	348.70	43.35	600.00
32.90	406.88	45.53	648.00
35.25	445.70	47.65	697.82
38.28	498.50	49.90	751.60

DeJong (17) described a Fortran IV program which tabulates the vapor pressure of halothane at temperatures of 15 to  $25^{\circ}\text{C}$  in  $0.1^{\circ}$  increments. An extensive data table was published (18) which includes vapor pressure and critical point data for halothane.

## 2.7 Density

The density of halothane at different temperatures was reported (TABLE I).

TABLE I

<u>temperature (<math>^{\circ}\text{C}</math>)</u>	<u>density (<math>\text{g}/\text{cm}^3</math>)</u>
0	$1.92336 \pm 4 \times 10^{-5}$ (19)
20	1.8677 (20)
25	$1.85612 \pm 3 \times 10^{-5}$ (19)
30	1.8446 (20)
35	$1.82820 \pm 3 \times 10^{-5}$ (19)
37	$1.82205 \pm 3 \times 10^{-5}$ (21)

An equation [ $d = d_0(1 - \alpha t)$ ] was given for calculating the density ( $d$ ) at any given temperature ( $t$ ,  $^{\circ}\text{C}$ ) knowing the density at  $0^{\circ}\text{C}$  ( $d_0$ ) and the temperature coefficient of expansion ( $\alpha$ ). A least squares analysis of the data gave:

$$d_0 = 1.9235 \pm 5 \times 10^{-4} \text{ and } \alpha = 1.411 \times 10^{-3} \text{ for halothane (19).}$$

## 2.8 Refractive Index

The refractive index of halothane was reported to be (20):

$$n_D^{20} = 1.3696 \qquad n_D^{30} = 1.3647$$

## 2.9 Solubility

The temperature coefficients of solubility of halothane (22) in blood, water, saline, oil and fat can be calculated from the following equations:

$$\begin{aligned} K_w &= 1.2750 \log_{10} \lambda_w - 3.3601 \\ K_o &= 1.2897 \log_{10} \lambda_o - 0.9389 \end{aligned}$$

where  $\lambda_w$  and  $\lambda_o$  are the Ostwald solubility coefficients for pure water and oil and  $K_w$  and  $K_o$  are the temperature coefficients of solubility (in percent change of solubility per degree Celsius) in water, blood or aqueous tissues and in olive oil or fat respectively. The solubility of halothane in blood or water (23-28), in lipids and lipid tissue (27,29), rubbers (29), the partition coefficient in octanol-water (30) and the partition coefficients in blood or water (26,31-33), tissue (33), oil (33) and rubber (33) have been reported. Ostwald solubility coefficients for Krebs' solution have been reported as 0.78 (25) and 0.75 (32).

### 2.10 Hydrates/Clathrates

The halothane-hydrogen sulfide mixed hydrate has been studied by NMR (34) and by mass spectroscopy (35) which showed that the crystal had a clathrate structure in which hydrogen sulfide, water and halothane did not interact chemically. The halothane-tri-o-thymotide clathrate was studied as a function of temperature by fluorine-19 NMR (36).

### 2.11 Miscellaneous

Heats of vaporization have been reported for the following temperatures:

$t, ^\circ\text{C}$	$\Delta H_v, \text{ kJ/mole}$
25	29.64
28	29.42
40	28.69
50	29.03
55	27.81
70	26.81

The critical temperature  $T_c (^{\circ}\text{K})$  was reported as  $496.3^{\circ}\text{K}$  and the heat of vaporization at the normal boiling point ( $50.07^{\circ}\text{C}$ ) as  $-28.09 \text{ kJ/mole}$  (20).

The Joule-Thompson coefficient has been reported as  $1.1808^{\circ}\text{C/Atm}$  (37).

The heat of formation has been reported as  $-690.4 \pm 4.9 \text{ kJ/mole}$  (38) and  $-691 \text{ kJ/mole}$  (39).

## 3. Synthesis and Purification

### 3.1 Synthesis

In addition to the methods listed in the original monograph, halothane can be prepared by the following routes: (j) hydrobromination of 1,1,2-trifluoro-2-chloroethylene followed by rearrangement in situ using aluminum trichloride or tribromide as a catalyst (40-43). The 1,1,2-trifluoro-2-chloroethylene can be generated by dechlorination of Freon 113(42); (k) selective debromination of 1,1,1-trifluoro-2,2-dibromo-2-chloroethane with an alcohol and base (44) or with 2-propanol and ultra-violet irradiation (45); (l) vapor



phase chlorination of 1,1,1-trifluoro-2-bromoethane with ultra-violet irradiation (46); (m) ethoxidation of 1,1,2-trichloroethylene followed by bromination and then hydrofluoridation using the sulfur tetrafluoride-hydrogen fluoride complex (47) or omitting the ethoxidation, 1,1,2-trichloroethylene can be reacted directly with bromine and then with fluorine (42).

Refinements of synthesis (i) -the rearrangement of 1,1,2-trifluoro-2-bromo-1-chloroethane with aluminum trichloride include the use of inhibitors to increase the yield from 84.9% up to 96.6% (48), in situ formed aluminum trihalide (49), and removal of halothane from the reaction under reduced pressure (50). A review of earlier methods was published by Scipioni and Gambaretto (51).

Of further interest is the synthesis of deuteriohalothane, tritiohalothane and halothane-1-C-14. Deutero- and tritiohalothane are prepared from halothane by the reaction (n) with deuterium oxide and tritium oxide in the presence of base (52,53), while halothane-1-C-14 is prepared (o) by fluoridation of 1-C-14-chloroacetic acid with sulfur tetrafluoride to give 1,1,1-trifluoroethyl chloride-1-C-14 followed by bromination to give the 1-C-14 labeled halothane (54).

Both optical isomers of halothane have been prepared (p) starting with resolved chlorobromoacetic acid followed by reaction with sulfur tetrafluoride. The resulting optically active isomers were 73-80% optically pure (14). These reactions are shown in Figure 1, where the letters correspond to the various methods mentioned above.

### 3.2 Impurities

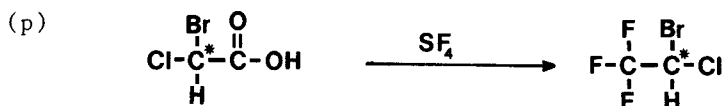
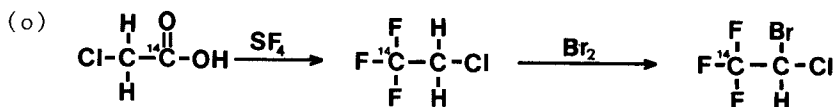
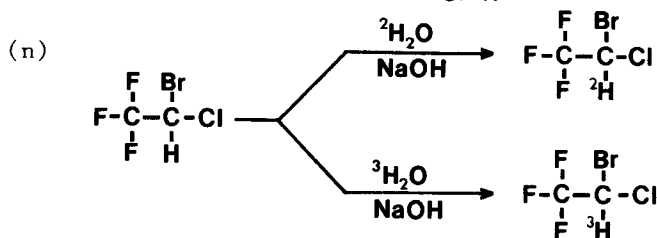
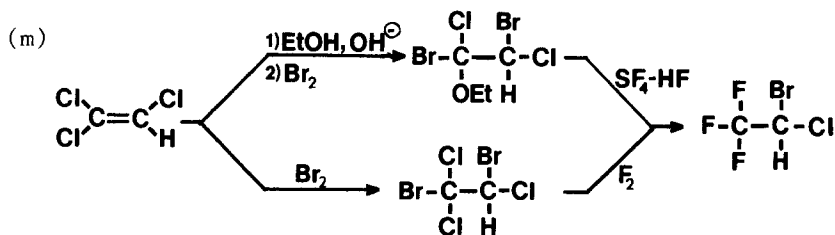
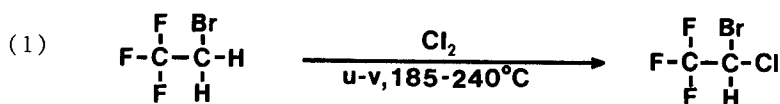
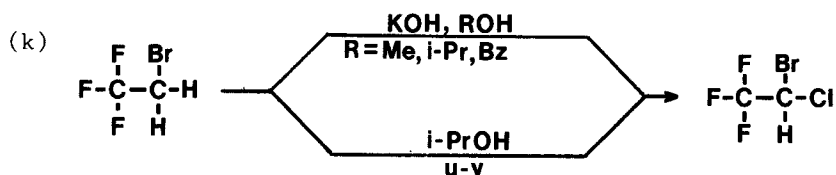
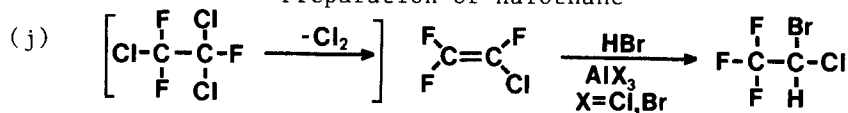
Besides those impurities listed in section 6.21 of the original monograph, the following impurities have been reported in halothane: 1,1,1-trichloro-2,2,2-trifluoroethane and 1,1,2-trifluoro-1-bromo-2-chloroethane (55); 1,2-dichlorohexafluorocyclobutane (56,58) and 1,1-difluoro-2-bromo-2-chloroethylene (57,58). A review of halothane impurities was published by Kodama (59).

### 3.3 Purification

Halothane impurities can be removed by azeotropic distillation with solvents such as acetone and tetrahydrofuran (56) or with excess starting material (43); by passage thru

Figure 1

## Preparation of Halothane



beds of molecular sieve (60), active alumina (61) or activated charcoal (62); by halogenation with chlorine (63) or bromine (64) or a halogenating agent such as aluminum trichloride or bromide or antimony pentachloride (65) followed by extraction with base and distillation (64); oxidation of impurities using common oxidizing agents (57,66); by treatment with amines such as piperidine, ethanolamine or pyrrolidine followed by distillation (67).

#### 4. Stability and Degradation

##### 4.1 Stability

Contact of halothane with heated steam humidifiers was found to result in the formation of five condensates (68). However, complete characterization and quantification could not be done. Addition of small amounts of  $C_1$  to  $C_4$  alcohols to halothane was reported to inhibit formation of acidic impurities on long storage (69). Butyraldoxime; 2,6-dimethylmorpholine; 2,4-xyleneol and eugenol were reported to be alternative antioxidants to thymol which occasionally causes the disruption of the anesthetic apparatus (70).

##### 4.2 Inflammability

Halothane was found to be nonflammable in oxygen under conditions encountered in clinical anesthesia (71,72). However, in high concentrations of nitrous oxide (65%) and oxygen (30%) halothane was found to be inflammable (71). Studies of the explosion limits of halothane and nitrous oxide with oxygen and air have been made and discussed (73).

##### 4.3 Degradation

Halothane has been found to degrade when passed over the carbon dioxide absorbant - soda lime - in closed circuit systems. The identified decomposition products were 1,1,1-trifluoro-2-chlorethane and 1,1-difluoro-2-bromo-2-chloroethylene (74). A common halothane impurity-1,1,2-trifluoro-1-bromo-2-chloroethane gave the toxic impurity 1,1,2-trifluoro-2-chloroethylene when used in conjunction with soda lime (55).

#### 5. Drug Metabolic Products

Bromide ( $Br^-$ ); fluoride ( $F^-$ ); trifluoroacetaldehyde (TFA); trifluoroacetyl chloride (TFAC); 2-chloro-1,1,1-trifluoroethane (CTE); 2-chloro-1,1-difluoroethylene (CDE); trifluoro-

acetic acid (TFAA); N-trifluoroacetyl-2-aminoethanol (TFAAE) and N-acetyl-S-(2-bromo-2-chloro-1,1-difluoroethyl)-L-cysteine (ABCDL) have been reported as metabolites of halothane in various species of animals and man. The following metabolites were found:

Man CTE,CDE(75); TFA,TFA(76); F<sup>-</sup>(77); TFAA (78); TFAAE(78);  
ABCDL(78)  
Rabbit CTE,CDE(79); Br<sup>-</sup>(80); TFAA(80)  
Rat CTE,CDE(81); F<sup>-</sup>(82)  
Dog F<sup>-</sup>(83)  
Horse Br<sup>-</sup>(84)

Trifluoroethanol has been postulated (85) as a possible metabolite, however, several authors (86-89) refuted this theory. In addition, several review articles have been published on the metabolites of halothane (90-94).

## 6. Methods of Analysis

### 6.1 Analysis of Halothane in Mixtures

#### 6.11 Gas Chromatography

Salvioni (95) reported the use of gas sampling bulbs and gas chromatography on Chromosorb 102 with flame ionization for determining halothane in operating rooms.

#### 6.12 Infrared Absorption

Zeller (96) and Lane (97) used infrared absorption to analyze halothane in operating rooms. Gedeon et al (98) described a fast response analyzer for on-line measurement of halothane. An infrared camera for measuring halothane in operating rooms was described by Carlsson et al (99).

#### 6.13 Ultraviolet Absorption

Several apparatuses have been described which measure halothane in gas streams by UV detection (100-104). They are mainly used for monitoring the concentration of halothane in the expired breath of patients undergoing gaseous anesthesia. To eliminate the interference of CO<sub>2</sub>, O<sub>2</sub>, N<sub>2</sub>, H<sub>2</sub>O vapor and N<sub>2</sub>O, a filter is used which cuts out the UV below 230 nm and allows the measurement of halothane between 240 and 260 nm (101).

#### 6.14 Other Methods

Matschiner et al (105) dehalogenated halothane electrochemically in acetic acid/ammonium acetate to give 80% 1-chloro-2,2-difluoroethylene. Middaugh et al (106) reported the formation of 1-chloro-2,2,2-trifluoroethane and 1,1,1-trifluoroethane by electrochemical reduction with half-wave potentials of 0.62 and 1.42 volts. Albery et al (107) and Gol'din et al (108) studied the polarographic behavior of halothane. The limiting current was found to be directly related to the halothane concentration (107) and bromide ions were produced (107).

A quantitative procedure was reported for the determination of halothane based on combustion in oxygen and quantitation of the bromide ions produced by argentometric titration and by direct potential measurement with an ion-selective electrode (109). Houdek and Kadlec (110) determined halothane by combustion and measurement of the decrease in combustion enthalpy of a fuel gas carrier. Leichnitz (111) combusted halothane and measured the halogen adsorbed in a detector tube.

Tremolieres (112) reported the use of an interferometer for the determination of halothane in oxygen.

Detectors for halothane based on resonance frequency changes of a coated quartz crystal have been described (113-115).

A colormetric procedure for halothane in air by absorption in ethanol and color development based on Fujiwara's reaction was reported (116).

X-ray spectrometry has been used to determine halothane adsorbed on charcoal (117,118). The detection limit using Br  $K_{\alpha}$  radiation was ca. 0.002 TLV hours and was linear for at least 30 hours (117).

An instrument (Katharometer) based on the thermal conductivity of a gas mixture was reported (119) which continuously measures the halothane concentration in a carrier gas.

An air-condenser detector for the determination of halothane in O-N<sub>2</sub>O was described (120,121). The monitor measures the dielectric constants of the gases.

Gas analyzers based on liquid crystals have been described for monitoring halothane during use in anesthesia (122-124).

Use of mass spectrometers for measuring halothane uptake and respiration in surgical patients was reported (125-129). The same technique was also applied to operating rooms (125). Graham et al (130) reported errors which occur in mass spectrometer monitoring during anesthesia.

## 7. Determination in Body Fluids and Tissues

### 7.1 Gas Chromatographic Methods

#### 7.14 Headspace Analysis

Gas chromatographic methods using headspace analysis for halothane and other volatiles in whole blood (131,132), plasma (131) and serum (131) have been reported.

#### 7.15 Mass Spectrometric Detection

Nagata et al (133) developed a gas chromatographic-mass spectrometric method for halothane in autopsy samples.

### 7.2 Absorptiometric Methods

#### 7.23 Ultraviolet Absorption

Blanck and Thompson (134) extracted samples with heptane and measured the halothane concentration at 208 nm.

#### 7.24 Nuclear Magnetic Resonance Spectroscopy (NMR)

A noninvasive multinuclear NMR technique is described by Wyrwicz et al (135) which permits extracorporeal observation of blood under in-vivo conditions for the determination of halothane.

### 7.4 Electrochemical

Albery and Hahn (136,137) developed an electrochemical sensor for halothane in fluids which consists of a Au or Pt electrode and a Ag electrode which are exposed to the fluid through a permeable membrane.

### 7.5 Liquid Scintillation

Smith et al (138) described a method by which C-14 labeled halothane was determined in blood samples by liquid scintillation counting.

### Acknowledgements

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# CUMULATIVE INDEX

Bold numerals refer to volume numbers

- Acetaminophen, **3**, 1; **14**, 551  
Acetohexamide, **1**, 1; **2**, 573  
Allopurinol, **7**, 1  
Alpha-tocopheryl acetate, **3**, 111  
Amantadine, **12**, 1  
Amikacin sulfate, **12**, 37  
Aminophylline, **11**, 1  
Aminosalicylic acid, **10**, 1  
Amitriptyline hydrochloride, **3**, 127  
Amoxicillin, **7**, 19  
Amphotericin B, **6**, 1; **7**, 502  
Ampicillin, **2**, 1; **4**, 518  
Ascorbic acid, **11**, 45  
Aspirin, **8**, 1  
Atenolol, **13**, 1  
Atropine, **14**, 32  
Azathioprine, **10**, 29  
Bacitracin, **9**, 1  
Baclofen, **14**, 527  
Bendroflumethiazide, **5**, 1; **6**, 597  
Benperidol **14**, 245  
Benzocaine, **12**, 73  
Benzyl benzoate, **10**, 55  
Betamethasone dipropionate, **6**, 43  
Bretylum tosylate, **9**, 71  
Bromocriptine methanesulfonate, **8**, 47  
Calcitriol, **8**, 83  
Camphor, **13**, 27  
Captopril, **11**, 79  
Carbamazepine, **9**, 87  
Cefaclor, **9**, 107  
Cefamandole nafate, **9**, 125; **10**, 729  
Cefazolin, **4**, 1  
Cefotaxime, **11**, 139  
Cefoxitin, sodium, **11**, 169  
Cephalexin, **4**, 21  
Cephalothin sodium, **1**, 319  
Cephradine, **5**, 21  
Chloral hydrate, **2**, 85  
Chloramphenicol, **4**, 47, 518  
Chlordiazepoxide, **1**, 15  
Chlordiazepoxide hydrochloride, **1**, 39; **4**, 518  
Chloroquine, **13**, 95  
Chloroquine phosphate, **5**, 61  
Chlorpheniramine maleate, **7**, 43  
Chlorprothixene, **2**, 63  
Chlortetracycline hydrochloride, **8**, 101  
Chlorthalidone, **14**, 1  
Cholecalciferol, *see* Vitamin D<sub>3</sub>  
Cimetidine, **13**, 127  
Cisplatin, **14**, 77  
Clidinium bromide, **2**, 145  
Clindamycin hydrochloride, **10**, 75  
Clofibrate, **11**, 197  
Clonazepam, **6**, 61  
Clorazepate dipotassium, **4**, 91  
Clotrimazole, **11**, 225  
Cloxacillin sodium, **4**, 113  
Codeine phosphate, **10**, 93  
Colchicine, **10**, 139  
Cyanocobalamin, **10**, 183  
Cyclizine, **6**, 83; **7**, 502  
Cycloserine, **1**, 53  
Cyclothiazide, **1**, 66  
Cyproheptadine, **9**, 155  
Dapsone, **5**, 87  
Dexamethasone, **2**, 163; **4**, 519  
Diatrizoic acid, **4**, 137; **5**, 556  
Diazepam, **1**, 79; **4**, 518  
Dibenzepin hydrochloride, **9**, 181  
Dibucaine and dibucaine hydrochloride, **12**, 105  
Diflunisal, **14**, 491  
Digitoxin, **3**, 149  
Digoxin, **9**, 207  
Dihydroergotoxine methanesulfonate, **7**, 81  
Dioctyl sodium sulfosuccinate, **2**, 199; **12**, 713

- Diperodon, **6**, 99  
Diphenhydramine hydrochloride, **3**, 173  
Diphenoxylate hydrochloride, **7**, 149  
Disopyramide phosphate, **13**, 183  
Disulfiram, **4**, 168  
Dobutamine hydrochloride, **8**, 139  
Dopamine hydrochloride, **11**, 257  
Doxorubicine, **9**, 245  
Droperidol, **7**, 171  
Echothiophate iodide, **3**, 233  
Emetine hydrochloride, **10**, 289  
Epinephrine, **7**, 193  
Ergonovine maleate, **11**, 273  
Ergotamine tartrate, **6**, 113  
Erythromycin, **8**, 159  
Erythromycin estolate, **1**, 101; **2**, 573  
Estradiol valerate, **4**, 192  
Estrone, **12**, 135  
Ethambutol hydrochloride, **7**, 231  
Ethynodiol diacetate, **3**, 253  
Etomidate, **12**, 191  
Fenoprofen calcium **6**, 161  
Flucytosine, **5**, 115  
Fludrocortisone acetate, **3**, 281  
Flufenamic acid, **11**, 313  
Fluorouracil, **2**, 221  
Fluoxymesterone, **7**, 251  
Fluphenazine decanoate, **9**, 275; **10**, 730  
Fluphenazine enanthate, **2**, 245; **4**, 524  
Fluphenazine hydrochloride, **2**, 263; **4**, 519  
Flurazepam hydrochloride, **3**, 307  
Gentamicin sulfate, **9**, 295; **10**, 731  
Glibenclamide, **10**, 337  
Gluthethimide, **5**, 139  
Gramicidin, **8**, 179  
Griseofulvin, **8**, 219; **9**, 583  
Halcinonide, **8**, 251  
Haloperidol, **9**, 341  
Halothane, **1**, 119; **2**, 573; **14**, 597  
Heparin sodium, **12**, 215  
Heroin, **10**, 357  
Hexestrol, **11**, 347  
Hexetidine, **7**, 277  
Hydralazine hydrochloride, **8**, 283  
Hydrochlorothiazide, **10**, 405  
Hydrocortisone, **12**, 277  
Hydroflumethiazide, **7**, 297  
Hydroxyprogesterone caproate, **4**, 209  
Hydroxyzine dihydrochloride, **7**, 319  
Imipramine hydrochloride, **14**, 37  
Indomethacin, **13**, 211  
Iodipamide, **2**, 333  
Iopanoic acid, **14**, 181  
Isocarboxazid, **2**, 295  
Isoniazide, **6**, 183  
Isopropamide, **2**, 315; **12**, 721  
Isoproterenol, **14**, 391  
Isosorbide dinitrate, **4**, 225; **5**, 556  
Kanamycin sulfate, **6**, 259  
Ketamine, **6**, 297  
Ketoprofen, **10**, 443  
Ketotifen, **13**, 239  
Khellin, **9**, 371  
Leucovorin calcium, **8**, 315  
Levallorphan tartrate, **2**, 339  
Levarterenol bitartrate, **1**, 49; **2**, 573; **11**, 555  
Levodopa, **5**, 189  
Levothyroxine sodium, **5**, 225  
Lidocaine base and hydrochloride, **14**, 207  
Lorazepam, **9**, 397  
Mefloquine hydrochloride, **14**, 157  
Melfalan, **13**, 265  
Meperidine hydrochloride, **1**, 175  
Meprobamate, **1**, 209; **4**, 520; **11**, 587  
6-Mercaptopurine, **7**, 343  
Mestranol, **11**, 375  
Methadone hydrochloride, **3**, 365; **4**, 520; **9**, 601  
Methaqualone, **4**, 245, 520  
Methimazole, **8**, 351  
Methotrexate, **5**, 283  
Methoxsalen, **9**, 427  
Methyclothiazide, **5**, 307  
Methylphenidate hydrochloride, **10**, 473  
Methypyrrol, **2**, 363  
Metoprolol tartrate, **12**, 325  
Metronidazole, **5**, 327  
Minocycline, **6**, 323  
Moxalactam disodium, **13**, 305  
Nabilone, **10**, 499  
Nadolol, **9**, 455; **10**, 732  
Nalidixic acid, **8**, 371  
Naloxone hydrochloride, **14**, 453  
Natamycin, **10**, 513  
Neomycin, **8**, 399  
Nitrazepam, **9**, 487  
Nitrofurantoin, **5**, 345  
Nitroglycerin, **9**, 519  
Norethindrone, **4**, 268  
Norgestrel, **4**, 294  
Nortriptyline hydrochloride, **1**, 233; **2**, 573  
Noscapine, **11**, 407  
Nystatin, **6**, 341  
Oxazepam, **3**, 441  
Oxyphenbutazone, **13**, 333  
Oxytocin, **10**, 563  
Penicillamine, **10**, 601

- Penicillin-G benzothine, **11**, 463  
Penicillin-V, **1**, 249  
Pentazocine, **13**, 361  
Phenazopyridine hydrochloride, **3**, 465  
Phenelzine sulfate, **2**, 383  
Phenformin hydrochloride, **4**, 319; **5**, 429  
Phenobarbital, **7**, 359  
Phenoxymethyl penicillin potassium, **1**, 249  
Phenylbutazone, **11**, 483  
Phenylephrine hydrochloride, **3**, 483  
Phenylpropanolamine hydrochloride, **12**, 357; **13**, 771  
Phenytol, **13**, 417  
Pilocarpine, **12**, 385  
Piperazine estrone sulfate, **5**, 375  
Primidone, **2**, 409  
Probenecid, **10**, 639  
Procainamide hydrochloride, **4**, 333  
Procarbazine hydrochloride, **5**, 403  
Promethazine hydrochloride, **5**, 429  
Proparacaine hydrochloride, **6**, 423  
Propiomazine hydrochloride, **2**, 439  
Propoxyphene hydrochloride, **1**, 301; **4**, 520; **6**, 598  
Propylthiouracil, **6**, 457  
Pseudoephedrine hydrochloride, **8**, 489  
Pyrazinamide, **12**, 433  
Pyridoxine hydrochloride, **13**, 447  
Pyrimethamine, **12**, 463  
Quinidine sulfate, **12**, 483  
Quinine hydrochloride, **12**, 547  
Reserpine, **4**, 384; **5**, 557; **13**, 737  
Rifampin, **5**, 467  
Rutin, **12**, 623  
Saccharin, **13**, 487  
Salbutamol, **10**, 665  
Salicylamide, **13**, 521  
Secobarbital sodium, **1**, 343  
Silver sulfadiazine, **13**, 553  
Sodium nitroprusside, **6**, 487  
Spironolactone, **4**, 431  
Succinylcholine chloride, **10**, 691  
Sulfadiazine, **11**, 523  
Sulfamethazine, **7**, 401  
Sulfamethoxazole, **2**, 467; **4**, 521  
Sulfasalazine, **5**, 515  
Sulfisoxazole, **2**, 487  
Sulindac, **13**, 573  
Sulphamerazine, **6**, 515  
Terpin hydrate, **14**, 273  
Testolactone, **5**, 533  
Testosterone enanthate, **4**, 452  
Tetracycline hydrochloride, **13**, 597  
Theophylline, **4**, 466  
Thiostrepton, **7**, 423  
Tolbutamide, **3**, 513; **5**, 557; **13**, 719  
Triamcinolone, **1**, 367; **2**, 571; **4**, 521; 524; **11**, 593  
Triamcinolone acetonide, **1**, 397, 416; **2**, 571; **4**, 521; **7**, 501; **11**, 615  
Triamcinolone diacetate, **1**, 423; **11**, 651  
Triamcinolone hexacetonide, **6**, 579  
Triclobisonium chloride, **2**, 507  
Trifluoperazine hydrochloride, **9**, 543  
Triflupromazine hydrochloride, **2**, 523; **4**, 521; **5**, 557  
Trimethaphan camsylate, **3**, 545  
Trimethobenzamide hydrochloride, **2**, 551  
Trimethoprim, **7**, 445  
Trimipramine maleate, **12**, 683  
Trioxsalen, **10**, 705  
Tripelennamine hydrochloride, **14**, 107  
Triprolidine hydrochloride, **8**, 509  
Tropicamide, **3**, 565  
Tubocurarine chloride, **7**, 477  
Tybamate, **4**, 494  
Valproate sodium and valproic acid, **8**, 529  
Vinblastine sulfate, **1**, 443  
Vincristine sulfate, **1**, 463  
Vitamin D<sub>3</sub>, **13**, 655  
Warfarin, **14**, 243  
Xylometazoline hydrochloride, **14**, 135

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